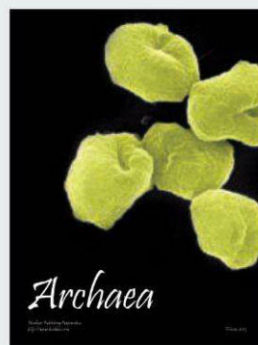
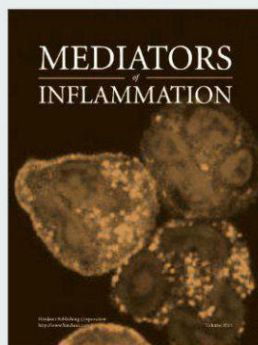
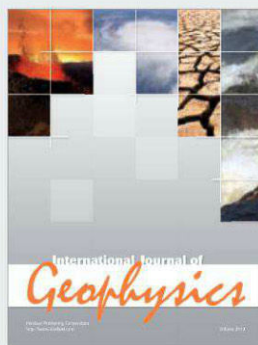
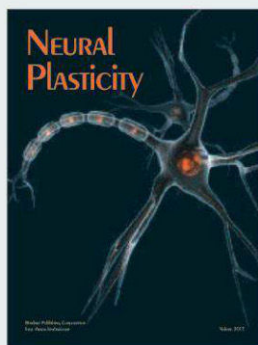
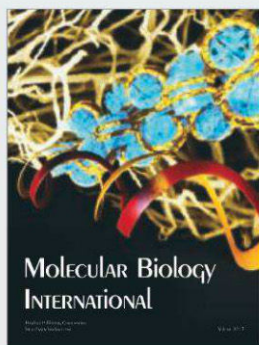
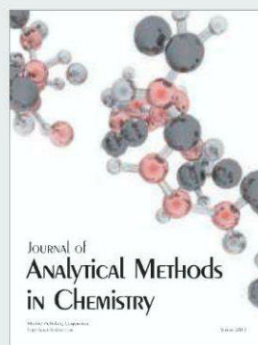
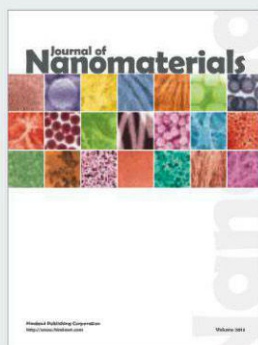
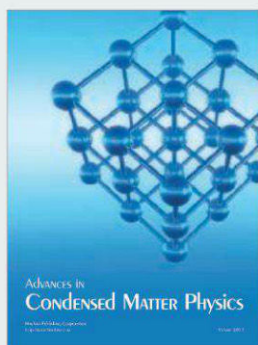
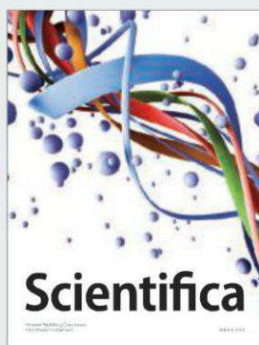
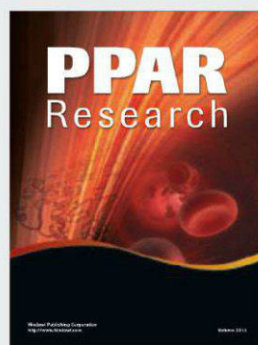
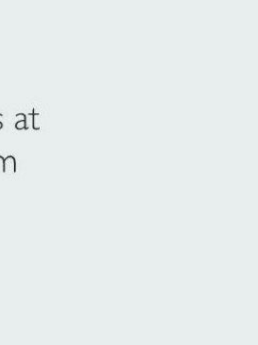
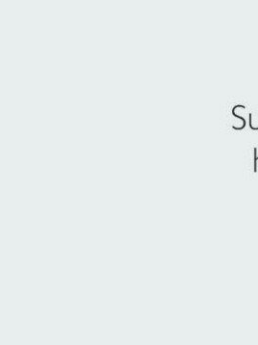
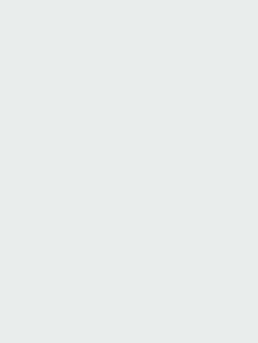
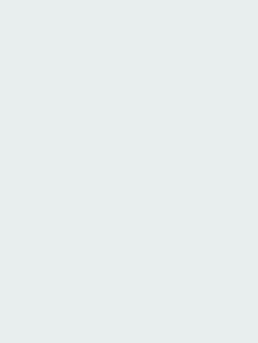
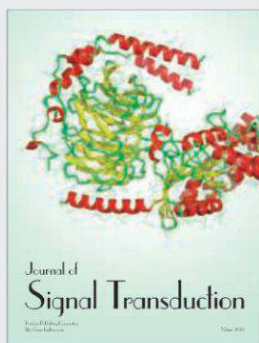
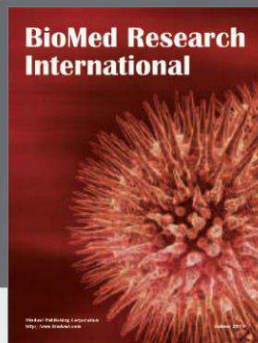
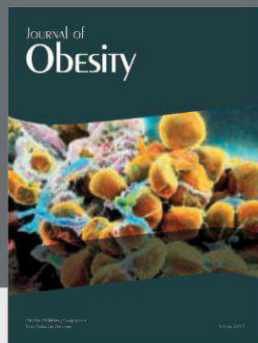
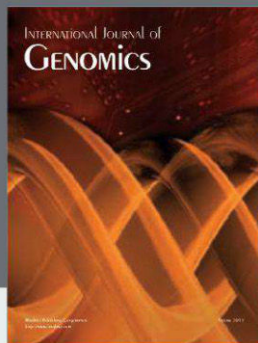
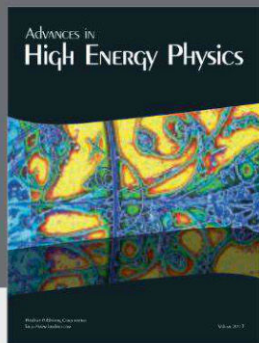




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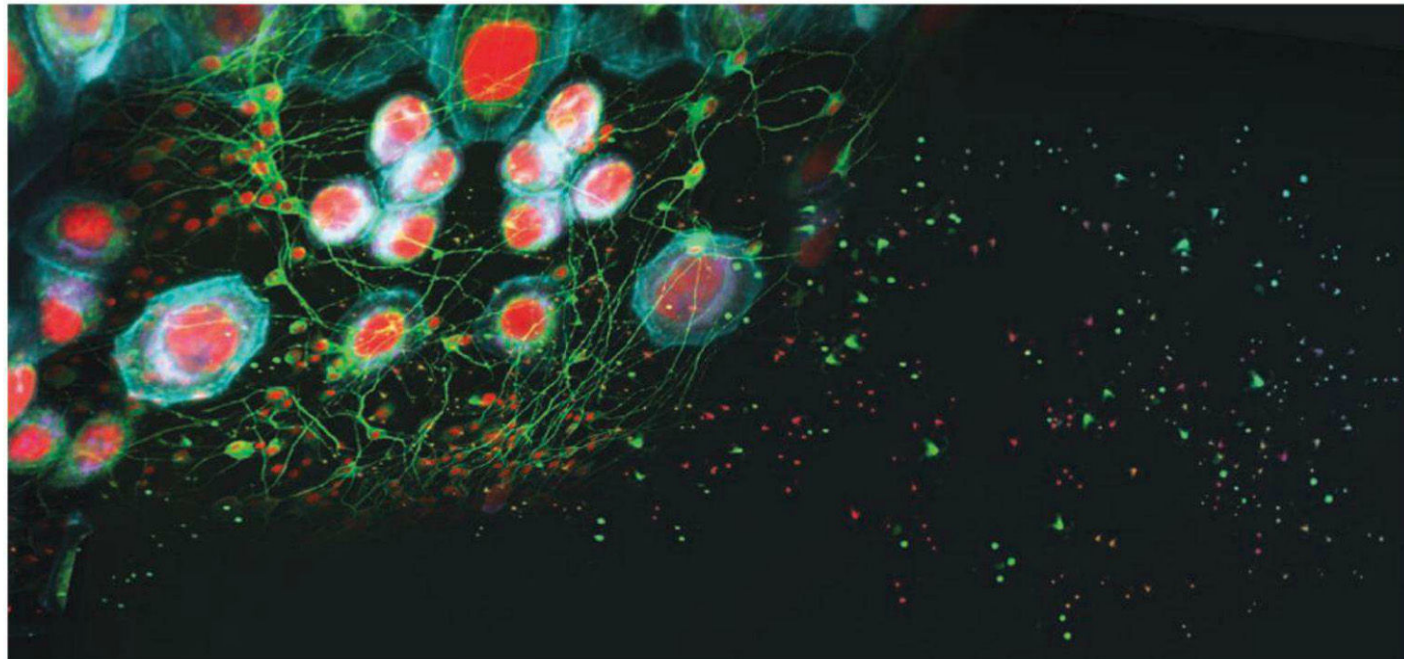
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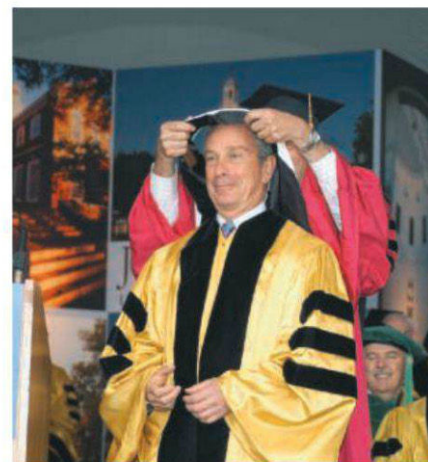
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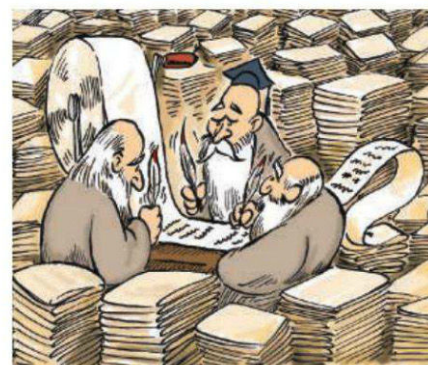
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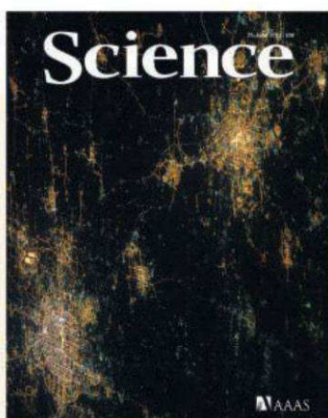
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COVER

View of Beijing and Tianjin, China, from the International Space Station. Though cities have different sizes and characteristics, mathematical theory can relate size and population density to social, spatial, and infrastructural properties. Such modeling provides a view of the nature of cities and may help urban planners determine how cities evolve and whether they have reached their full potential. See pages 1418 and 1438.

Image: Image Science and Analysis Laboratory, NASA-Johnson Space Center

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- 1421** Pliocene Warmth, Polar Amplification, and Stepped Pleistocene Cooling Recorded in NE Arctic Russia

J. Brigham-Grette et al.

A sediment core from Lake El'gygytyn, in northeast Russia, provides a high-latitude climate record of the late Pliocene.

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B. Hunt et al.

A band gap is observed in a monolayer graphene–hexagonal boron nitride heterostructure.

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- 1431** Engineering Coherence Among Excited States in Synthetic Heterodimer Systems

D. Hayes et al.

Small molecules comprising bridged chromophores manifest a quantum mechanical effect observed in light-harvesting proteins.

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- 1434** Direct Imaging of Covalent Bond Structure in Single-Molecule Chemical Reactions

D. G. de Oteyza et al.

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- 1438** The Origins of Scaling in Cities

L. M. A. Bettencourt

Cities of all sizes can be modeled as interdependent networks of interactions and infrastructure.

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- 1442** Topology of Feather Melanocyte Progenitor Niche Allows Complex Pigment Patterns to Emerge

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- 1445** Protein Equilibration Through Somatic Ring Canals in *Drosophila*

P. F. McLean and L. Cooley

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- 1448** Quantum Coherent Energy Transfer over Varying Pathways in Single Light-Harvesting Complexes

R. Hildner et al.

A phase relation observed in ensemble measurements of photosynthetic proteins is borne out at the single-molecule level.

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- 1456** GPR15-Mediated Homing Controls Immune Homeostasis in the Large Intestine Mucosa

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- 1459** H5N1 Hybrid Viruses Bearing 2009/H1N1 Virus Genes Transmit in Guinea Pigs by Respiratory Droplet

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- 1463** An Airborne Transmissible Avian Influenza H5 Hemagglutinin Seen at the Atomic Level

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- 1467** GWAS of 126,559 Individuals Identifies Genetic Variants Associated with Educational Attainment

C. A. Rietveld et al.

Three genetic loci are found to explain variation associated with educational achievement.

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- 1472** BigBrain: An Ultrahigh-Resolution 3D Human Brain Model

K. Amunts et al.

A freely available microscopic model of human brain architecture with a spatial resolution of 20 micrometers is presented.

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- 1475** Compartmentalized Calcium Transients Trigger Dendrite Pruning in *Drosophila* Sensory Neurons

T. Kanamori et al.

During fruit fly metamorphosis, dendritic calcium signaling defines the branches to be eliminated in sensory neurons.



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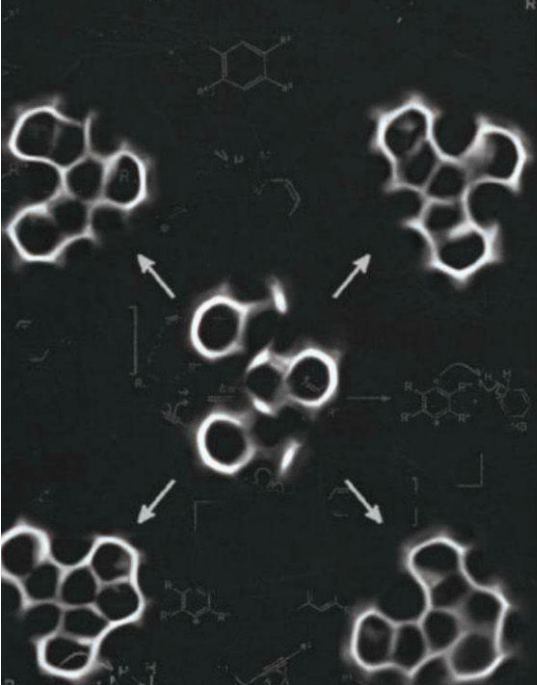
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Watching Organic Reactions

Single-molecule studies can overcome the difficulty of inferring the various outcomes of reactions in ensemble measurements. **De Oteyza et al.** (p. 1434, published online 30 May; see the Perspective by **Giessibl**) used a variation of noncontact atomic force microscopy in which the imaging tip was derivatized with a single CO molecule to obtain subnanometer-resolution images of conjugated organic molecules undergoing reaction on a silver surface. Different thermally induced cyclization reactions of oligo(phenylene-1,2-ethynylenes) were observed.

Furthering Exfoliation

In addition to graphene, a wide range of layered materials, including oxides, chalcogenides, and clays are of interest because of their optical, electrical, and mechanical properties. While many methods can be used to cleave layered sheets from the bulk material, they are difficult to scale up. Liquid exfoliation routes may hold the best promise for making materials in large quantities. **Nicolosi et al.** (p. 1420) review progress in developing exfoliation routes, both aqueous and nonaqueous for a wide range of starting materials.

From Russia with Lovely Data

Climate and the atmospheric concentration of CO₂ are closely linked. **Brigham-Grette et al.** (p. 1421, published online 9 May) present data from Lake El'gygytyn, in northeast Arctic Russia,

that shows how climate varied between 3.6 and 2.2 million years ago, an important interval in the global cooling trend that accelerated rapidly at the end of the Miocene. Summer temperatures were about 10°C warmer than today, even though the concentration of atmospheric CO₂ was similar.

Coherence in Photosynthesis

It is unclear how energy absorbed by pigments in antenna proteins is transferred to the central site of chemical catalysis during photosynthesis. **Hildner et al.** (p. 1448) observed coherence—prolonged persistence of a quantum mechanical phase relationship—at the single-molecule level in light-harvesting complexes from purple bacteria. The results bolster conclusions from past ensemble measurements that coherence plays a pivotal role in photosynthetic energy transfer. **Hayes et al.** (p. 1431, published online 18 April) examined a series of small molecules comprised of bridged chromophores that also manifest prolonged coherence.

GPR15 Gets T_{regs} to Guard the Gut

The large intestine is the site that is typically most inflamed in Crohn's disease and ulcerative colitis, which are thought to result when the immune system is not able to keep the peace with trillions of resident gut microbes. The immune system does this by recruiting specific cell populations, like regulatory T cells (T_{regs}), to the gut. **Kim et al.** (p. 1456, published online 9 May) now suggest that the orphan G protein-coupled receptor GPR15 is expressed by T_{regs} and required for T_{reg} homing to the large intestine in mice.

Influencing Influenza

Currently, there is anxiety that the avian H5N1 influenza virus will reassort with the highly transmissible and epidemic H1N1 subtype to trigger a virulent human pandemic. **Y. Zhang et al.** (p. 1459, published online 2 May) used reverse genetics to make all possible reassortants between a virulent bird H5N1 with genes from a human pandemic H1N1. Virulence was tested in mice and transmissibility was tested between guinea pigs, which have both avian- and human-like airway influenza virus receptors. To assess what is happening to the receptor-ligand interactions as a result of these mutations, **W. Zhang et al.** (p. 1463, published online 2 May) probed the structure of both wild-type and mutant hemagglutinin of H5

in complex with analogs of the avian and human receptor types. Certain mutations in the receptor-binding site changed binding affinity.

The Equations Underlying Cities

Cities are complex systems of which functioning depends upon many social, economic, and environmental factors. **Bettencourt** (p. 1438; see the cover; see the Perspective by **Batty**) developed a theory to explain the quantitative relationships observed between various aspects of cities and population size or land area.

Genetic College

Many genomic elements in humans are associated with behavior, including educational attainment. In a genome-wide association study including more than 100,000 samples, **Rietveld et al.** (p. 1467, published online 30 May; see the Perspective by **Flint and Munafò**) looked for genes related to educational attainment in Caucasians. Small genetic effects at three loci appeared to impact educational attainment.

Reconstructing the Human Brain

Reference brains have become a standard tool in human brain research. Reference brains presently in the public domain provide a spatial framework at the macroscopic level. **Amunts et al.** (p. 1472) present a high-resolution (20 μm)



three-dimensional reconstruction of a human brain. The tool will be freely available to help with interpreting functional neuroimaging studies, fiber tract analyses, and assigning molecular and gene expression data.

Dendritic Pruning

During metamorphosis, *Drosophila* sensory neurons eliminate their dendritic trees, but axons and soma remain intact. **Kanamori et al.** (p. 1475, published online 30 May) demonstrate that compartmentalized calcium transients in dendrites function as the spatiotemporal cue for pruning of unwanted branches. Such a localized calcium signal, induced by a local elevation of branch excitability, activates calcium-dependent proteinases and eventually causes branch death.

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Keith R. Yamamoto is a professor in the Department of Cellular and Molecular Pharmacology at the University of California, San Francisco. He cochaired the *ARISE II* committee. E-mail: yamamoto@cmp.ucsf.edu.

Time to Play Ball

WHAT IF BASEBALL WERE ORGANIZED LIKE SCIENCE? ASPIRING CATCHERS OR SHORTSTOPS, LIKE students of physics or molecular biology, would be trained by professional counterparts, and top prospects with dazzling skills would turn pro without learning that by combining their specialized talents, they could create an entirely different game. Managers, owners, and marketers would seek and reward individual stars at each position but would not facilitate or nurture a team culture or even a team game. In science, traditions, policies, and bureaucracies isolate scientific disciplines and their discoveries and technologies, squandering exciting opportunities that could be empowered by merged ideas and efforts—in short, by teamwork. A recent report from the American Academy of Arts and Sciences asserts that knocking down such boundaries would reveal great new opportunities; indeed, a new game.

The report, *ARISE II: Unleashing America's Research & Innovation Enterprise*,* crafted by a committee from academia and the private sector, sees the scientific endeavor today as daunted by the challenge of extracting understanding from floods of disconnected data that threaten to swamp every discipline. To achieve understanding demands unprecedented levels of integration along two separate but intersecting planes. One plane embodies deep collaboration across the physical and life sciences, from basic discovery through the many branches of development and application. It is a call for a new synthesis, reaching well beyond the shared facilities and multidisciplinary programs that are now quite common, toward entities in which the expertise and imagination of researchers and practitioners from separate fields synergize to achieve "transdisciplinarity." The other plane of integration involves the major stakeholder sectors in the scientific enterprise: academia, government, private industry, and nonprofit organizations. They must move beyond ad hoc "deals" and establish policies, training programs, and mechanisms that bring together people, knowledge, and resources across current stakeholder boundaries.

How might these lofty goals be achieved? The committee advanced 11 specific recommendations. One calls for a revolutionary computational "knowledge network," expanding on a 2011 National Research Council report.[†] This continuously evolving information commons—an electronic resource—would recognize and display links between approaches, findings, and investigators in different fields and sectors, suggesting unrecognized hypotheses or predictions, and "self-assembling" potential teams of collaborators that can address issues that might not otherwise even have been formulated.

To promote synergies among the stakeholder sectors, "grand challenges" were proposed at sufficient scale and scope to capture public imagination, strike creative sparks among both individual investigators and self-assembled teams across the continuum, and incentivize support and participation from multiple stakeholders. Grand challenges seem to be in style these days, albeit at modest scales compared to those envisioned here, but some may provide preliminary glimpses of multistakeholder buy-in. Other recommendations reach toward the broad goals of the report: Overhaul academic promotion policies, devise transdisciplinary curricula in which students learn and work in teams, establish technology transfer mechanisms that prioritize knowledge exchange over revenue, create policies that acknowledge and manage conflicts of interest rather than claim to eliminate them, and incentivize cooperation among government agencies. Again, isolated efforts in some of these directions are under way. What is needed is a coordinated strategy. Universities, companies, and private entities that work cooperatively will themselves benefit, and in turn provoke others to join. The impact on science and society could be transformative, not just nationally but globally. Implementing the recommendations of *ARISE II* may not yield a *Field of Dreams*, but everyone will get a better chance to play ball.

— Keith Yamamoto

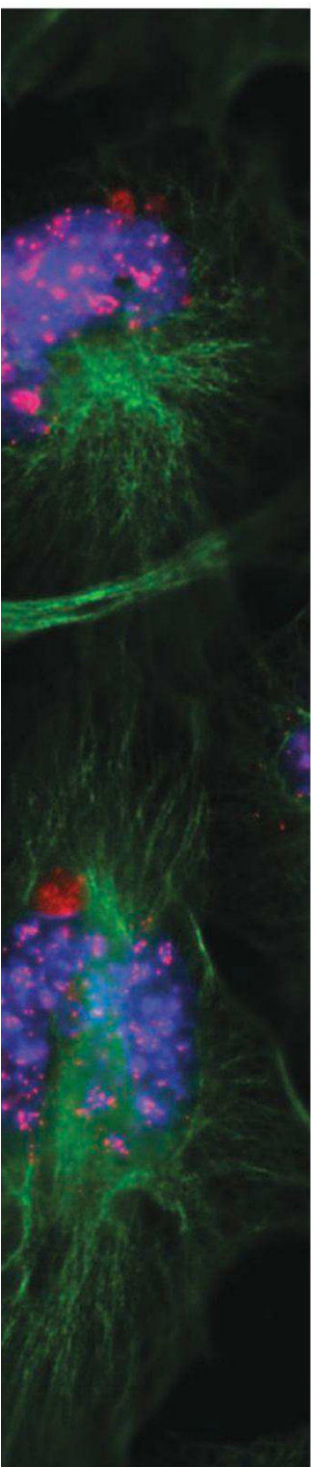
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*www.amacad.org/arise2/. [†]http://www.nap.edu/catalog.php?record_id=13284.

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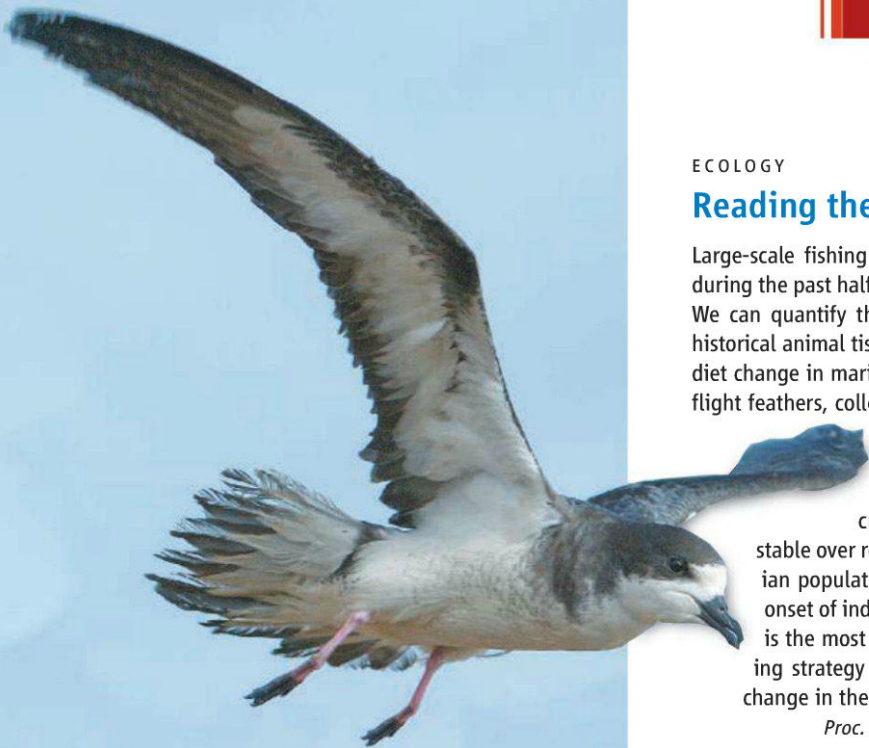
ECOLOGY

Reading the Bones

Large-scale fishing impacts in the open ocean are recent, mostly occurring during the past half century as industrial fishing technologies were developed. We can quantify these impacts on marine food webs by using current and historical animal tissues to document the temporal signature of human-driven diet change in marine species. Wiley *et al.* looked at isotopes from bones and flight feathers, collected at modern and historical breeding sites, of Hawaiian

petrels. Ranging from current to 4000 years old, specimens represented individuals alive well before the first humans arrived on Hawaii, as well as those from the current depleted system. The percentage of N^{15} was relatively stable over roughly 3000 years, but rapidly decreased, across all Hawaiian populations, within the past 50 to 100 years, coincident with the onset of industrial fishing. A fisheries-induced decrease in trophic level is the most likely cause of this shift, and the petrel's generalist foraging strategy further suggests the presence of a wider fisheries-driven change in the oceanic food webs of the northeast Pacific Ocean. — SNV

Proc. Natl. Acad. Sci. U.S.A. **110**, 10.1073/pnas.1300213110 (2013).



ECONOMICS

Patent Commons

The ability of "green tech" to tackle environmental challenges depends on both scientific advances and deployed technologies. Despite widespread use of intellectual property (IP) incentives to promote technology transfer, IP restrictions may stifle diffusion of knowledge and development of "green" innovations. One proposed solution is the Eco-Patent Commons (EPC), formed in 2008 by IBM, Sony, Nokia, and Pitney Bowes. Participating firms offer, for free use by anyone, some of their patented environmentally relevant technologies. Hall and Helmers found that although EPC technologies were typically environmentally friendly, many were tangential to the creation of new environmentally sound technology. EPC patents were less than 0.1% of EPC firms' overall patent portfolios but were of similar value to the firms' other, non-EPC patents. Compared to other patents (including many from non-EPC firms), the EPC patents were of lower value. While acknowledging that EPC is relatively new and that knowledge diffusion may take years, they found that free access has not yet increased EPC patent citations. — BW

J. Environ. Econ. Manag.

10.1016/j.jeeem.2012.12.008 (2013).

CELL BIOLOGY

Ultralong Antibodies

An antibody's specificity for antigens is largely determined by complementarity-determining regions (CDRs). These form protein loops of

the heavy and light chains that bind to a target. The most diverse is region 3 of the immunoglobulin heavy chain (CDR H3), which forms a flat binding surface for antigens. However, 10% of all bovine antibodies bear a much longer loop that includes many cysteines. Wang *et al.* analyzed the crystal structures of two such cow antibodies and discovered that the stretch of residues forms a long stalk that protrudes from the antibody and terminates in a knob domain. The two antibodies have different disulfide bond patterns in the knob, which arise from different cysteine sequence positions. The stalks also differ in length, which alters the relative position of the knob domain. Sequencing of more than 10,000 ultralong CDR H3s indicates that the diversity (D) gene segment appears primed for mutation to cysteine, allowing new disulfide bonds to be made or broken. The authors propose that the cow's immune system uses cysteine diversification to increase the repertoire of unique "minifolds" in knob domains, thereby expanding the antibody arsenal for recognizing foreign targets. — LC

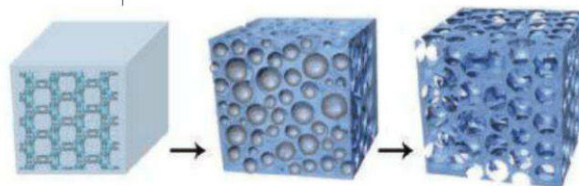
Cell **153**, 10.1016/j.cell.2013.04.049 (2013).

MATERIALS SCIENCE

From MOFs to Mesoporous Oxides

Mesoporous oxides can be made starting with organic frameworks, such as micelles, that form a template for metallorganic precursors. After the precursors react to form the oxide, the template can then be removed. Kim *et al.* have used crys-

talline metal organic frameworks (MOFs) as the precursors for magnesium and cerium oxides that develop mesoscale porosity. They made the MOF precursors with adipic acid, $\text{HOOC}(\text{CH}_2)_4\text{COOH}$, as the linker. The solvothermal reaction of a magnesium or cerium salt with this ligand and methanol and dimethylacetamide created MOFs in which each metal ion was coordinated to five or six oxygen atoms. Heating these materials under a flowing nitrogen atmosphere to 500°C and then holding these materials at that temperature for 12 hours created crystalline oxides.



These oxides had a hierarchical structure of mesopores on the scale of tens of nanometers connected by larger pores on the scale of 50 to 100 nm. The magnesium oxide material also exhibited a high capacity for adsorption of carbon dioxide (~9% by weight) from combustion gas. The authors argue that the thermal decomposition process creates organic species that act as micellar templates ("porogens") that are removed during the thermal treatment. Thermal treatment of a MOF made with a more stable aromatic linker did not form the mesoporous oxide but instead led to nanoparticle formation. — PDS

J. Am. Chem. Soc. 10.1021/ja401869h (2013).

Continued on page 1379

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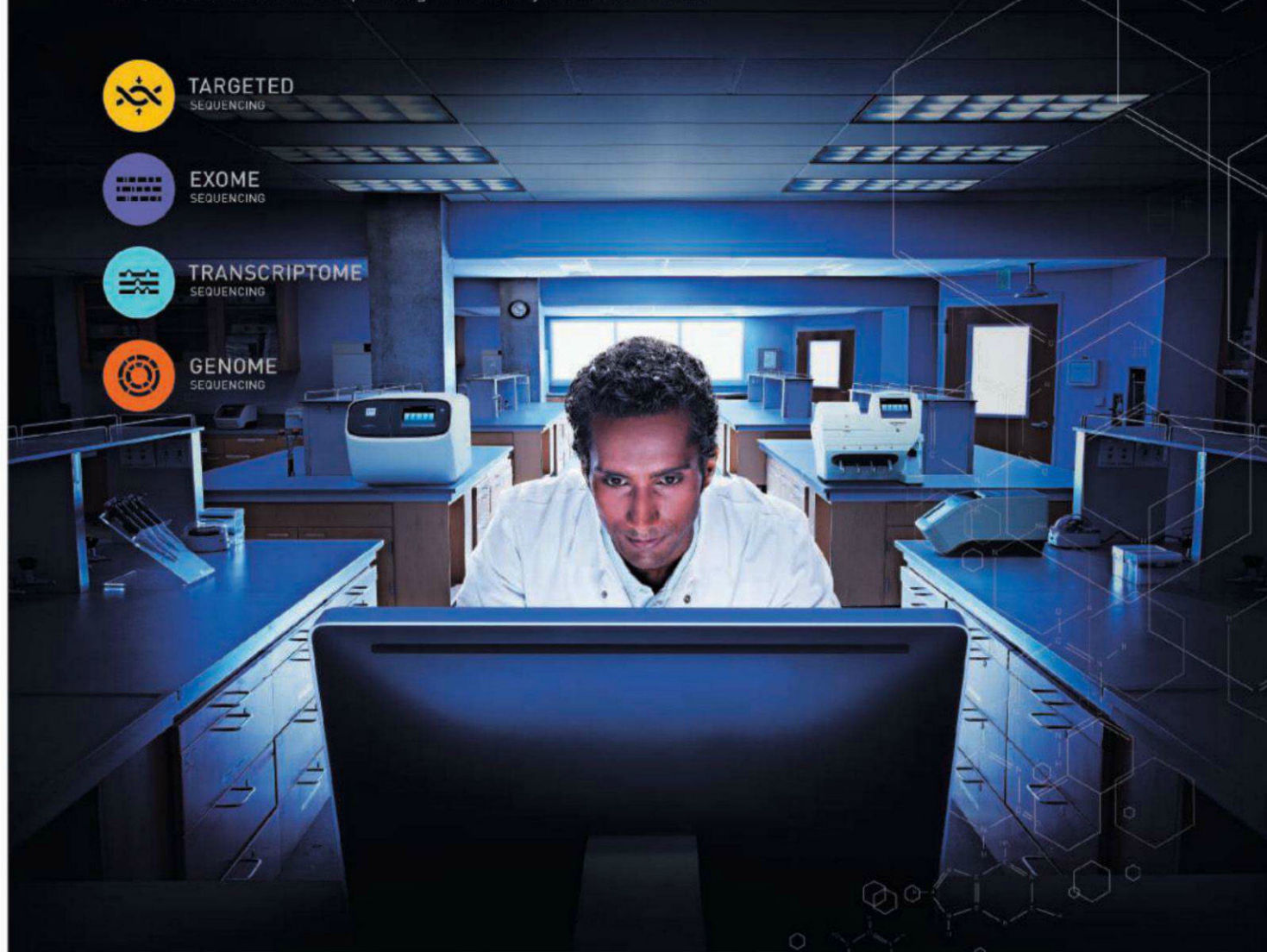
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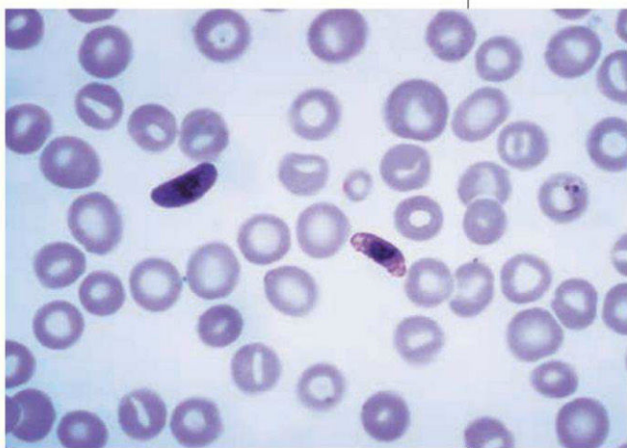
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Continued from page 1377

MICROBIOLOGY

Tuning Haploid Happenstance

How can the malarial parasite *Plasmodium falciparum* evolve drug resistance when it is haploid and does not have recourse to meiosis? Guler *et al.* challenged *P. falciparum* with a drug nicknamed DSM1, which inhibits the parasite's flavonoid enzyme, dihydroorotate dehydrogenase (DHODH), required for the biosynthesis of pyrimidine and nucleic acids. Resistant clones arose that had multiple copies of DHODH and no point mutations. Deep sequencing revealed that an initial duplication arose stochastically by mitotic rearrangements between scattered tracks of polyadenine or polythymine (A/T). The rigidity of A/T tracks is known to trigger recombination and so could promote mitotic DNA rearrangements. Hence, the first step is luck, and eventually a beneficial mutation is selected out of the millions of failures in the replicating parasite population. Subsequently, copies of DHODH are precisely ramped up and down depending on drug pressure. The amplicon responsible for resistance was the only new amplicon seen in DSM1-resistant parasites, so there is a further mechanism for restraining the collateral damage arising from



unlimited amplification of bits of haploid genome. In the A/T-rich genome of *P. falciparum*, this two-step mechanism may be the primary driver for evolution, not just drug resistance. — CA

PLoS Pathogens 9, 10.1371/journal.ppat.1003375 (2013).

GEOPHYSICS

Spinning Iron in the Mantle

Transitions in the spin state, or electronic configuration, of iron can be induced by the high temperatures and pressures in Earth's interior. Spin transitions—whether they be abrupt or gradual with depth—could influence

the physical properties of abundant iron-bearing minerals or affect the interpretation of seismic data; however, laboratory experiments at such extreme conditions have often been contradictory. Wu *et al.* developed a simple theoretical formulation to simulate the iron spin transition and used it to calculate elasticity in ferropericlase [(Mg, Fe)O], a major mineral in the lower mantle, across a wide range of pressures and iron compositions. The calculations predict that the spin transition should not have a strong effect on some important elastic properties, such as the shear modulus (the ratio of shear stress to shear strain). Moreover, the concentration of iron in ferropericlase will strongly influence seismic wave velocities, requiring readjustment of expected mantle compositions that are based on extrapolations of experimental results. — NW

Phys. Rev. Lett. 110, 228501 (2013).

PHYSICS

Flat Condensate

Ultracold gases of atoms, which can be cooled to temperatures close to absolute zero, are a promising model system for the collective phenomena that emerge in the presence of many particles. To keep the atoms together, atomic physicists use “traps,” with the center of the trap being the most densely populated with atoms and the density trailing off toward the edges; this spatial non-uniformity, however, complicates comparison with many-body theories. Gaunt *et al.* created a nearly uniform trapping potential by using three laser beams (one “tube” and two “sheet” beams) and loaded it with

bosonic ^{87}Rb atoms, which they cooled down sufficiently to achieve their condensation into the lowest energy state. Both the condensed atoms and those that continued to populate higher energy levels behaved as one would expect from an ultracold gas in a uniform potential; for example, a signature bimodal distribution of momenta emerged with cooling below the transition temperature, whereas the spatial profile showed little change. It is expected that this technique will enable obtaining benchmark results for many-body theories and can be used to trap fermionic atoms as well. — JS

Phys. Rev. Lett. 110, 200406 (2013).



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Madrid 1

Protesters to Government: 'Save Spanish Science'

Spanish researchers arrived at a closed gate at the Ministry of Economy and Competitiveness in Madrid on 14 June, where they had wanted to deliver a message to their government: Save Spanish science. Their march was one of 19 protests in cities across Spain against recent cuts in research budgets and delays in allocating govern-



ment money. Protesters carried a 10-point wish list, signed by 45,000 supporters, that includes increasing national spending for research to 2% of the gross domestic product by 2016, improving opportunities for young researchers, and creating an independent funding agency.

The demonstrators wanted to deliver their petition at the ministry, which is responsible for science; when reporters weren't allowed to accompany them inside, they taped it to the gate instead. <http://scim.ag/Spanishprot>

Tokyo 2

Japan Considers Its Own NIH

Japan's government may create its own version of the U.S. National Institutes of Health (NIH) to foster medical innovation and bridge the gap between laboratory and clinic. The proposal is included in a draft Growth Strategy approved on 12 June by the administration's Industrial Competitiveness Council. The plan notes that a "Japanese NIH" could better meld governmental, academic, and private sector efforts "to strongly support the commercialization of innovative medical technologies." The new agency would be expected to "formulate a comprehensive strategy and prioritize goals and research targets for medical R&D."

The plan also calls for giving the existing Council for Science and Technology Policy a stronger hand so that it can better coordinate research efforts across ministries. It also mentions a host of measures—including changes to patent laws and tax incentives—intended to help turn research at national labs and universities into economic gains and to support private R&D. <http://scim.ag/JapanNIH>

Washington, D.C. 3

Proposed Status Bump For Captive Chimps

The U.S. Fish and Wildlife Service (FWS) last week announced a proposal to upgrade the status of captive chimpanzees from "threatened" to "endangered," the status that wild chimpanzees have had since 1990. The proposed rule would create a new permitting process for scientific research with captive chimpanzees, which would include the sale



across state lines of chimpanzee cell lines, tissue, or blood. If FWS finalizes the rule, scientists would receive permits only if their work aimed to "enhance the propagation or survival" of the species.

Scientists conducting biomedical studies with chimpanzees say that the rule change would do little to further conservation efforts and would hamper important research. "I am deeply disappointed," said John VandeBerg, chief scientific officer at the Texas Biomedical Research Institute in San Antonio, which has one of the largest colonies of captive chimpanzees available for research.

The need for chimpanzees in biomedical research came under close scrutiny in January by the Council of Councils at the U.S. National Institutes of Health (NIH), the main funder of such studies. In the next few weeks, NIH Director Francis Collins is expected to follow the council's recommendations and significantly curtail future support for captive chimpanzee research. <http://scim.ag/FWSchimps>

Woods Hole, Massachusetts and Chicago 4
MBL and University of Chicago Join Forces

The University of Chicago (UC) and the Marine Biological Laboratory (MBL) on 12 June announced an affiliation that will help keep MBL—the oldest private marine laboratory in the United States—afloat. Under the agreement, the Woods Hole, Massachusetts-based lab will remain an independent nonprofit and the University of Chicago will become the sole member of its corporation on 1 July, making the university ultimately responsible for MBL's budget. All MBL staff members will remain employees of the laboratory. The move comes after several years of falling revenues at MBL. According to tax documents, the private lab saw an 18% drop in income between 2008 and 2011. In 2011, the organization posted revenues of \$47.5 million—and a shortfall of \$798,000. Although the lab has done well in winning federal grant money over the last few years, the organization does not

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receive tuition funding and its alumni scientists haven't been big contributors. "It's not a sustainable business model anymore," says Joan Ruderman, MBL's president and director. Scientists at both organizations also see opportunities for collaboration in areas such as neuroscience, evolutionary and developmental biology, cell biology, and ecosystems science. <http://scim.ag/MBLChicago>

Baltimore, Maryland 5

More Eyes for 'Invisible' Trials

Publish your data, or we will—that's the warning to drug companies from Peter Doshi, a postdoctoral fellow at Johns Hopkins University in Baltimore, Maryland, and his colleagues. They want to convince researchers and journals to print unpublished data that is essentially privately held—but has become publicly available, such as through litigation or Freedom of Information Act requests. For example, Doshi's group at Hopkins has 178,000 pages of data on various drugs, many obtained from litigation against drug companies. An effort by the European Medicines Agency to share clinical trials data upon request led to the release of 1.9 million pages (since curtailed by lawsuits).

Doshi's team calls its proposal RIAT, for Restoring Invisible and Abandoned Trials. It was published on 13 June in *BMJ* and endorsed by *PLOS Medicine*. The authors propose several steps: Those interested in publishing the data should first notify the

drug company behind the research. If the company declines, those holding the documents should contact a RIAT-friendly journal about publishing the work themselves. While some may consider this "equivalent to intellectual property theft," the authors write, "you cannot steal what is already in the public domain." <http://scim.ag/RIATprop>

NEWSMAKERS

Transport Studies, Earth Modeling Earn Blue Planet Prizes

The Blue Planet Prize, which recognizes research addressing environmental problems, will go this year to climatologist **Taroh Matsuno**, now at the Japan Agency for Marine-Earth Science and Technology, for leading the development of the Earth Simulator, a supercomputer tailored for work on climate change; and to engineer **Daniel Sperling**, of the University of California, Davis, for opening new fields of study into more efficient and environmentally friendly transportation systems. Each man will receive \$527,000 at an October ceremony in Tokyo.



Matsuno

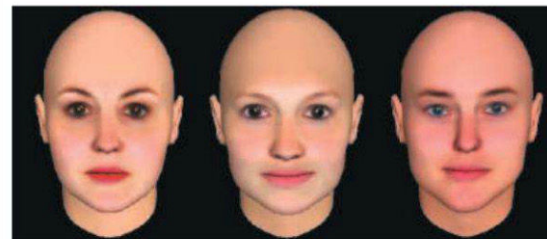


Sperling

FINDINGS

Beer Goggles for Your Brain

Hot? Or not? The lightning-quick spark that triggers desire when you see an attractive face is kindled within the ventral midbrain, associated with processing reward. Now, researchers have discovered a way to stoke that fire ... with 2 milliamps of electrical current.



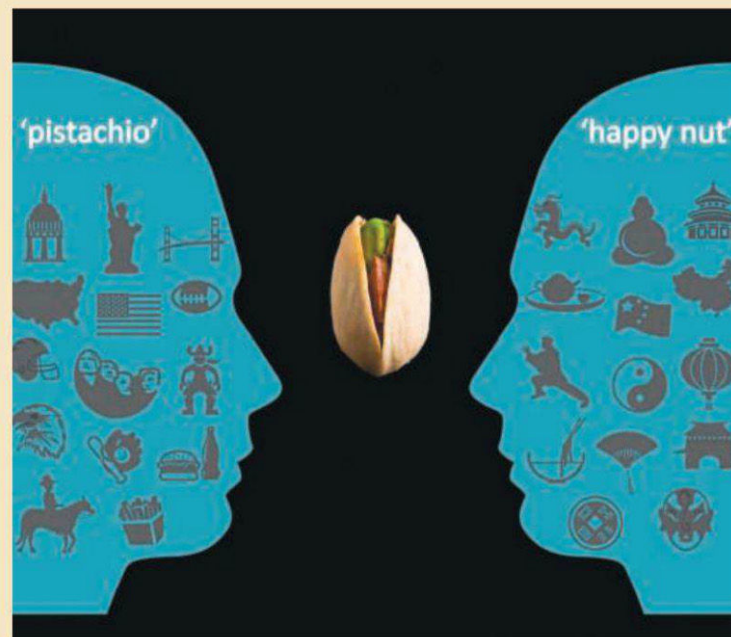
The research teams asked 19 volunteers to rate the attractiveness of two sets of computer-generated male and female faces with neutral expressions (examples above) before and after the activity in their ventral midbrains was ramped up using a technique called transcranial direct current stimulation (tDCS), which passes current through the brain between two electrodes on the scalp. A control group did the same, while receiving "sham" electrical stimulation that produced a tingling sensation but no real current. Compared with the control group, the volunteers who received tDCS rated the second set of faces as significantly >>

Memories of Home Delay Learning New Language

Reminders of home can hinder an immigrant's ability to speak a new language, suggests a new study by Columbia Business School psychologist Michael Morris and colleagues. The findings could help explain why cultural immersion is the best way to learn a foreign tongue and why immigrants who settle in ethnic enclaves acculturate more slowly.

To determine how cultural icons affect language, the researchers recruited Chinese students who had lived in the United States for less than a year. They sat opposite a computer displaying the face of "Michael Lee," either a Chinese or Caucasian male. Lee spoke English.

The team compared the fluency of the volunteers' English when talking to a Chinese versus a Caucasian face. Participants reported a more positive experience chatting with the Chinese Michael, but were significantly less fluent, producing 11% fewer words per minute on average, the authors report online on 17 June in the *Proceedings of the National Academy of Sciences*. And when asked to tell a story while viewing an image of the Great Wall, they were 85% more likely to use literal translations from Chinese for an object rather than the English term—for example, "happy nuts" instead of pistachio. <http://scim.ag/langremind>



>>FINDINGS

more attractive than the first set, the scientists reported online last week in *Translational Psychiatry*.

Similar techniques, the researchers say, could be used to treat disorders associated with faulty ventral midbrain circuitry, such as Parkinson's disease and schizophrenia—without drugs or invasive surgery.

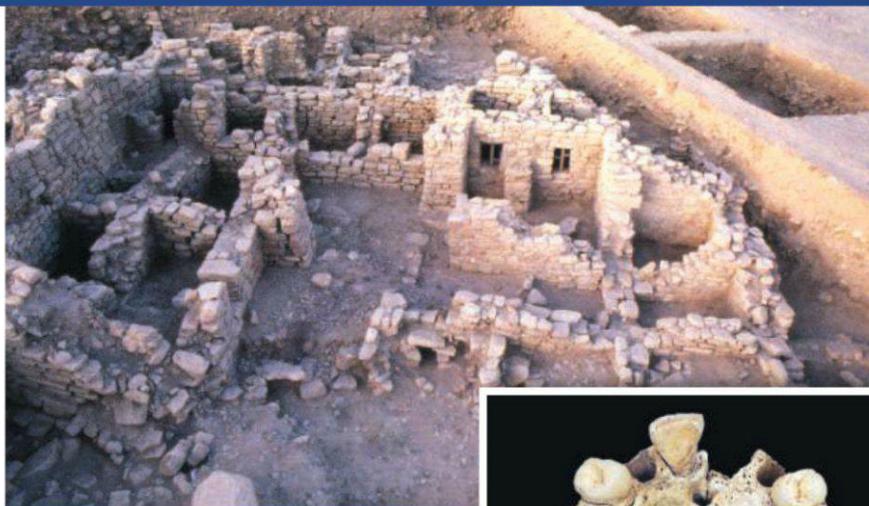
Did Inbreeding Bind Early Farmers Together?

About 10,000 years ago, roving hunter-gatherers in the Near East began settling down to form farming villages. What were the social ties that bound them into communities? A German team working at the 9500-year-old early farming site of Basta in Jordan has one answer: The inhabitants apparently engaged in inbreeding, although not necessarily incest.

The evidence for this startling conclusion, reported online last week in *PLOS*

Science LIVE

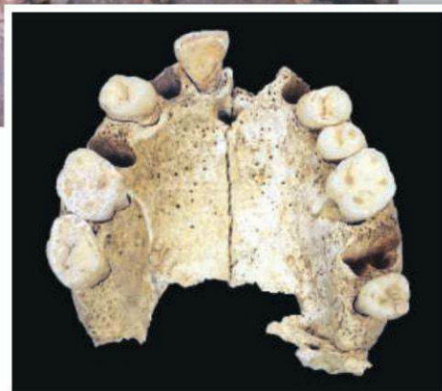
Join us on Thursday, 27 June, at 3 p.m. EDT for a live chat with experts on a **hot topic in science**. <http://scim.ag/science-live>



Tell-tale teeth. A high percentage of early farmers from Basta are missing two of their upper incisors (*inset*) due to inbreeding.

ONE, comes from a rare genetic anomaly in which both incisors are missing from the upper jaw. The incidence ranges from 0.5% to 3.0% in today's human populations, but it was 35.7% in 28 buried skeletons with preserved upper jaws. Even in groups known from their genealogy to have engaged in intensive inbreeding, this proportion never exceeds 20%, the team notes.

Many artifacts found at the site, including stone tools and jewelry, came from other farming sites in the Near East, a sign that the inhabitants traded widely. That means



inbreeding was a deliberate social choice rather than the result of geographic isolation, the team concludes. Despite hints of inbreeding at other sites, the researchers say that it's too early to tell if this social system helped create the ties that bound other farming villages together. <http://scim.ag/BastaInbreed>

Random Sample

Ocean Models Help Swimmer Navigate Florida Straits

Many endurance swimmers have an eye on the treacherous, tantalizing waters between Cuba and Florida. Australian swimmer Chloe McCardel's 12 June attempt to cross the Florida Straits was not the first—but she had a secret weapon: oceanography.

In 2012, University of Miami meteorologist Villy Kourafalou heard about a previous swimmer's unsuccessful attempt to be the first woman to swim the 170-kilometer distance unaided. Penny Palfrey's problem was clear, Kourafalou says: She was thwarted by shifting swirls, called eddies, spawned by the Florida current as it flows through the straits. Success, Kourafalou realized, may be all in the timing: Depending on ocean conditions on a given day, the eddies can either give a swimmer a boost or push her back. And that, Kourafalou adds, is how modeling could help McCardel. "We wanted her to know the circulation she's going to encounter," she says.

The Florida-based forecasting service ROFFS, which guides researchers, fishing expeditions, and commercial vessels through the straits, was also interested. "The current dominates the course rather than the swimmer," says founder Mitchell Roffer. "It's like a snake trapped between



two walls, constantly wiggling and changing its shape." ROFFS provided McCardel's team with high-resolution current models and streaming satellite data of surface ocean conditions, including infrared and water color imaging that show the density of plankton.

Using the models, the team selected a 12 June departure date, and McCardel set out. But her swim was cut short just 11 hours later—through no fault of physical oceanography, but after "debilitating" stings from jellyfish.



Endgame. A hearing on Myriad Genetics's *BRCA* gene patents drew protesters.

INTELLECTUAL PROPERTY

Supreme Court Rules Out Patents on 'Natural' Genes

Myriad Genetics, the Salt Lake City firm that built a \$450-million-a-year business on gene analysis, lost a big patent fight on 13 June. The U.S. Supreme Court gutted five of its intellectual property claims on the *BRCA1* and *BRCA2* genes, whose DNA sequences company scientists analyze to find mutations that raise a woman's risk of developing breast and ovarian cancer.

Few doubt that Myriad will survive the blow, but the 9–0 ruling against Myriad more broadly overturned 3 decades of U.S. support for human gene patents, rattling patent lawyers and biotech executives. The opinion, written by Justice Clarence Thomas, said that, "Myriad did not create anything. To be sure, it found an important and useful gene, but separating that gene from its surrounding genetic material is not an act of invention." Raw DNA sequences like these cancer genes, even if "isolated" from tissue, are a "product of nature," according to the court. They are not patentable.

It's the same argument put forward by Myriad's opponents, a coalition of doctors and researchers who wanted to break the

company's 15-year monopoly on *BRCA* testing. (Myriad charges more than \$3000 per test.) The challengers received legal help from the American Civil Liberties Union and an advocacy group called the Public Patent Foundation in New York City.

But the decision was not all in the coalition's favor. The court also ruled that human genes in the lab-made format known as complementary DNA, or cDNA, should remain patentable. The mixed signal seemed to confuse the stock market: On the morning of the decision, Myriad's stock shot up more than 11%. In the afternoon, it fell below the starting point and has remained there. Myriad still has the means to protect its investment, though: It holds 24 valid patents around *BRCA* testing, notes spokesperson Ronald Rogers, and it is entitled to sue anyone who infringes them.

Last week's ruling drew mixed responses from researchers and business leaders. Jim Greenwood, CEO of the Biotechnology Industry Organization in Washington, D.C., praised the allowance of cDNA patents. But he deplored the main outcome as a "depar-

tent stem cells would be in because they're actively created by adding genes.

The Supreme Court's rationale is "troublesome," says Carl Gulbrandsen, managing director of the Wisconsin Alumni Research Foundation, the nonprofit in Madison that holds the first hESC patents on behalf of the university. "It wouldn't surprise me if we got challenged on this." He argues that hESCs are not naturally occurring because they can be sustained only in carefully managed lab cultures. If they are unpatentable, Gulbrandsen says, the same logic would rule out patents on human insulin, human cell receptors, warfarin, enzymes, bacteria, microbes, and many other biological entities.

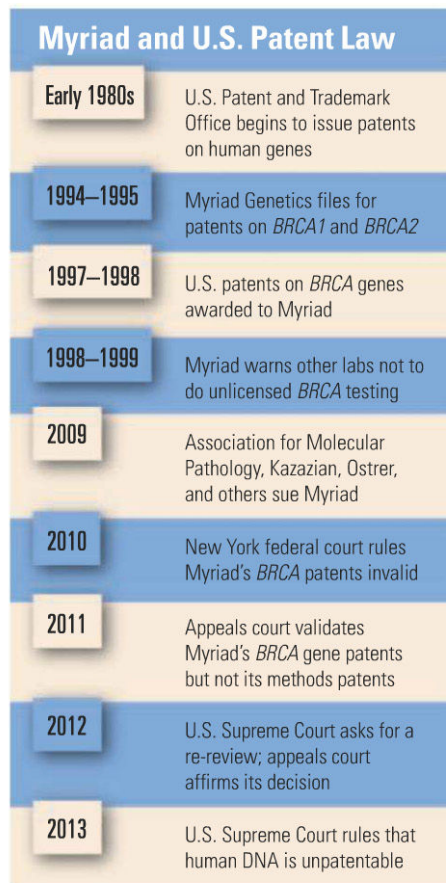
Many clinicians and scientists have been energized by the Supreme Court's logic. "It's a great decision," says Haig Kazazian Jr., a geneticist at Johns Hopkins University in Baltimore, Maryland, who provoked Myriad's ire a decade ago by testing the *BRCA* status of women worried about breast cancer, without seeking its permission. Myriad threatened to sue for infringement and "shut us down" in 1999, he says.

Although Myriad sued a number of private outfits, the lab under Kazazian and Arupa Ganguly, both then at the University of Pennsylvania (Penn), was the rare operating academic lab that it sought to halt. But Myriad also sent a warning to geneticist Harry Ostrer, formerly at New York University and now at the Albert Einstein College of Medicine in New York City, who had planned to do *BRCA* sequencing. Ostrer was sending specimens from his office to Penn for testing.

Ostrer received a warning letter from Myriad in 1999, according to court records, and stopped. In 2009, he signed up to sue Myriad over its *BRCA* patents and became the sole plaintiff in the case that went to the Supreme Court. Ostrer's "thrilled" with the outcome and says he is again planning to offer *BRCA* testing. The court has made it clear, he says, that, "inventors are going to have to really invent and not just extract."

After the verdict came down, many labs said they may add *BRCA* genes to the list of those they check, because it's less likely that Myriad will sue. These include, among others, groups at Penn; the University of Washington, Seattle; Quest Diagnostics; GeneDx; and Ambry Genetics (whose website carries an image of the Supreme Court with the banner, "Your Genes Have Been Freed").

On another front, Robert Nussbaum, a geneticist at UC San Francisco, is asking clinics around the world to send him and his colleagues the results of *BRCA* mutation analyses that they've ordered for patients over the years. He plans to put the anonymous details about mutations—originally provided to clients by Myriad—in a database to be shared on the Internet; he has



already received 4000 submissions. The Genetic Alliance, a patient advocacy group, aims to create a medical outcomes database with input from patients who were tested for *BRCA* genes.

Francis Collins, director of the National Institutes of Health and a champion of free access to DNA data, was ebullient. "Our position all along has been that patenting DNA in its natural state does not provide any benefit to the public," Collins said. "We

can breathe a big sigh of relief that [lawsuits] will no longer threaten to inhibit the progress of DNA research."

But Myriad found something to be happy about, too. Company spokesperson Rogers called last week's ruling "a very positive decision." That's because it gives new legal support to gene patents based on cDNA, which is a standard format for describing genes in most labs. Although the genetic sequence may be exactly the same, the court ruled that cDNA is patentable because it is lab-made, whereas DNA is not.

"We feel we are in a good position," says Myriad's Rogers, because the company still has 500 valid claims related to *BRCA* gene testing. "These won't go away" as a result of the ruling, Rogers says.

Because the primary *BRCA* patents are due to expire in any case in 2 years, Myriad was already getting ready to revamp its testing. The firm will discontinue its patented BRACAnalysis product and wrap the *BRCA* genes into a screening service that looks at an array of 25 risky genes.

Some patent attorneys, however, were discouraged. The U.S. patent system is the envy of the industrialized world but is "under attack," says Mercedes Meyer, a Ph.D. virologist and patent attorney for Drinker Biddle & Reath in Washington, D.C. "It is not broken," but is being turned into "Swiss cheese" thanks to decisions like the court's ruling on DNA. Meyers thinks that the legal situation on what is patentable for biotech firms has only become "muddier."

If that's correct, at least the patent lawyers have something to look forward to: more biotech litigation.

—ELIOT MARSHALL

With reporting by Michael Price.

HUMAN GENETICS

Agency Nixes deCODE's New Data-Mining Plan

The controversial company known for mining the DNA of Iceland's population to find links between genes and diseases, deCODE genetics, has hit another rough patch. A national agency that oversees data privacy in Iceland has rejected a request from deCODE to allow it to apply computational methods to the country's genealogical records to estimate the genotypes of 280,000 Icelanders, who have never agreed to take part in the company's research and link the data to hospital records. The Data Protection Authority (DPA) ruled that the company must first obtain individuals' informed consent.

The 28 May ruling, which deCODE is challenging, revives concerns about the practices of the company, which has pioneered population studies that link genes to risks for common diseases. A decade ago, an Icelandic court ruled that the company could not create and mine a database of the entire country's medical records without getting consent from individuals. With its latest research, the company appears to once again be trying to expand its studies to the entire population, this time by linking real or estimated genotypes for all 320,000 Icelanders to selected health records it is

acquiring. DPA's decision has also raised questions about several papers that the company has recently published using the strategy, and the company is taking heat in the Icelandic media.

Led by founder and CEO Kári Stefánsson, deCODE set out in 2006 to combine Iceland's extensive genealogical records, which it put into a database, with genetic data and also health records for all citizens. Unless individuals explicitly asked to opt out, all Icelanders would be included, making it one of the largest such biobanks in the world. The company would collect DNA from con-

sending individuals and mine this information to find disease genes.

After Iceland's Supreme Court said that deCODE could not use the health records without consent, deCODE instead built a research database using DNA and clinical data for more than 120,000 research volunteers and analyzed their DNA for a selection of subtle genetic variations called single-nucleotide polymorphisms (SNPs). The company has published a slew of papers in top journals tying specific genetic mutations to risks of diseases such as heart disease, diabetes, and cancer. deCODE's drug discovery efforts were less successful, and it filed for bankruptcy 4 years ago. Last December, however, Amgen purchased the company for \$415 million; it is now an Amgen subsidiary.

The current dispute involves a relatively new approach in which geneticists calculate the odds of whether an individual carries a particular genetic variant without directly sequencing their DNA. deCODE has done costly whole genome sequencing on DNA from only about 2500 research participants, but using the approach it has extended that data's reach to many more. When it finds a variant of interest among the whole genomes, the company can use the more limited SNP data that it has on tens of thousands of volunteers to impute, or infer, with 99% accuracy whether these individuals also carry the mutation. In this way, researchers can see if a disease risk variant found by fully sequencing the DNA of a small group holds up in a larger population.

deCODE is taking this technique a step further than others have, however. Combining the known and estimated genotypes for its research participants with its genealogical database, the company is also estimating what it calls in silico genotypes of close relatives of the SNP-chipped volunteers. That encompasses about 200,000 living and 80,000 dead Icelanders, who have not consented to participate in deCODE's studies, and would essentially give the company genotypes for the entire population. The firm then uses these estimated genotypes for individuals as controls in its studies and also combines them with health records for patients who are part

of a disease study in Iceland but whose DNA has not been sampled.

But when deCODE last November asked DPA if it could continue this strategy as part of a new study—it now wanted to impute the genotypes of close relatives of the SNP-chipped volunteers and then collaborate with researchers at Iceland's National Hospital to link them to certain hospital records for individuals, such as surgery codes and prescriptions—the oversight agency turned the company down. If deCODE wants to impute genotypes for these 280,000 living

to review the published papers.

Stefánsson argues that informed consent should not be necessary to link the health data to in silico genotypes derived from family trees because they are estimates, not directly measured DNA sequences, and are not very accurate for individuals, although they are valuable at the group level. "These are just conjectures about people, hypotheses," Stefánsson says. In his view, "they are not personal information."

Several geneticists contacted by *Science* say that it is unclear whether deCODE

should get informed consent. In a sense, deCODE is not doing anything new: To find genetic defects underlying rare familial diseases, geneticists have routinely inferred whether relatives who aren't part of the study carry the defective gene. deCODE is extending this strategy to the entire Icelandic population, notes University of Iceland geneticist Jón Jóhannes Jónsson. He opposed deCODE's original proposal to use national health records for genetic research without informed consent, but in this case, he's not sure "where you draw the line."

It's difficult to define."

Other geneticists, such as Daniel MacArthur of Massachusetts General Hospital in Boston, suggest that although deCODE does not seem to have violated the privacy of individuals, from an ethics standpoint its researchers should at least attempt to obtain informed consent.

Opinion is divided on how big a blow DPA's decision, should it stand, will be for deCODE's research. "In these studies, numbers are always a big advantage. But I don't think it's a deal breaker for their research if they can't [use the imputed genotypes]" because the company would still have data for more than 100,000 Icelanders, says University of Michigan, Ann Arbor, geneticist Goncalo Abecasis.

MacArthur disagrees: "Blocking deCODE from this imputation approach would greatly reduce the usefulness of their unique data set—that would be a tragedy for the company, but also for the wider complex disease genetics community."

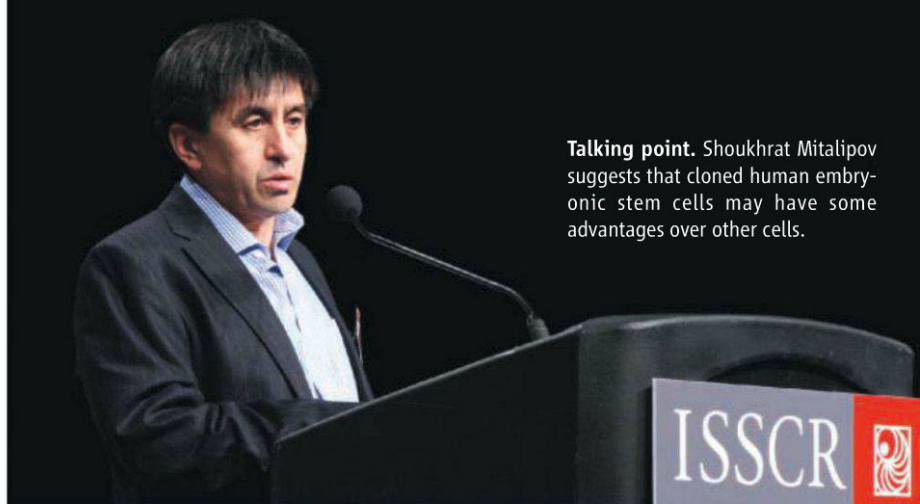
—JOCELYN KAISER



Fighting back. deCODE's Kári Stefánsson says Iceland's Data Protection Authority has reversed its position.

and dead relatives and link to their hospital data, it must obtain their informed consent, the DPA decision says. It gives the company until 1 November to present "documents on how the company has complied with the instructions."

Stefánsson is crying foul. He says that DPA, as well as Iceland's national bioethics committee, have until now approved the use of imputed genotypes for the two-thirds of Icelanders who have not consented to its research. deCODE has published six such studies on diseases such as gout and Alzheimer's in the last 2 years in *Nature* and other leading journals. "We have had approvals from both [bodies] for all of our studies," he says. "It makes little sense to us," he says. One newspaper in Iceland has suggested that the journals should consider retracting the papers in light of DPA's ruling. DPA interim Director Hörður Helgi Helgason tells *Science* that the agency's board will consider deCODE's request that it reevaluate the decision at a 25 June board meeting; for now, the agency has no plans



Talking point. Shoukhrat Mitalipov suggests that cloned human embryonic stem cells may have some advantages over other cells.

CELL BIOLOGY

Does Cloning Produce Better Embryonic Stem Cells?

BOSTON—For the past month, Shoukhrat Mitalipov has experienced the ups and downs of being a scientific celebrity. Widely lauded at first for accomplishing a long-sought feat, the production of cloned human embryonic stem (ES) cells, the reproductive biology specialist at Oregon Health & Science University in Beaverton was soon on the defensive for mistakes his team had made in the paper on the work that had been rushed into publication (*Science*, 31 May, p. 1026).

Colleagues have so far given him the benefit of the doubt, despite memories of a South Korean team fraudulently claiming the same victory nearly a decade ago. Mitalipov last week sought to allay any remaining fears by laying out his evidence at the annual meeting of the International Society for Stem Cell Research. Perhaps more provocative, he also showed the first data hinting that his cloned human ES cells may trump another kind of stem cell now in vogue, induced pluripotent stem (iPS) cells. Mitalipov said that compared with iPS cells, his cells bear “fewer abnormalities” and “more closely resemble genuine embryonic stem cells,” traits that may favor their use to study and perhaps treat particular conditions, such as diseases of a cell’s energy-producing mitochondria.

But the ability of other researchers to verify these advantages and exploit them may be thwarted by a thicket of regulations that limit the study or use of the embryo-derived cells, Mitalipov warned the audience. Indeed, when he sought to validate his team’s feat with a California scientist, he says that he was constrained to sending her only “digital data,” genetic analyses of the cells, rather than the actual cells.

That’s because his collaborator, Louise Laurent of the University of California, San

Diego, needs approval from a special university committee that oversees ES cell work to directly work with the cells. (Many universities created such regulatory bodies in response to a 2005 recommendation from the National Academies.) Other researchers may also be stymied from examining Mitalipov’s cells if they receive funding from the California Institute for Regenerative Medicine (CIRM), which prohibits the use of paid egg donors. Mitalipov created the cloned ES cells by putting skin cell nuclei into enucleated eggs—a procedure called somatic cell nuclear transfer (SCNT)—provided by women who were compensated. Mitalipov (not a CIRM grantee) received a letter from CIRM saying that it doesn’t allow grantees to study such cells.

“We knew that these cells were not able to go for NIH funding,” says Mitalipov, referring to the U.S. National Institutes of Health’s prohibition on using cells taken from embryos that are created solely for research. “But we had hoped CIRM funding could be used.”

In his talk, Mitalipov discussed four key cell types that his group has examined and compared: a widely studied skin cell line derived from a fetus; traditional ES cells created through in vitro fertilization (IVF); SCNT cells (produced by fusing the nuclei from the fetal skin cells and enucleated donor eggs); and iPS cells (formed by adding a cocktail of several genes to the fetal skin cells to make them behave like ES cells). Looking to corroborate his team’s results, Mitalipov conducted a genomic analysis of all the cell types and sent that data to Laurent, a stem cell specialist and obstetrician. Her analysis supported his findings, confirming that the SCNT cells carried nuclear DNA

identical to that of the fetal skin cells, but also mitochondrial DNA shared by the egg donor. This indicated that Mitalipov’s group had indeed transferred the nucleus from the skin cell into the cytoplasm of a donor egg, which contained its own, different mitochondria.

Mitalipov has since gone on to compare the SCNT cells to the iPS cells using a technique that analyzes patterns of methylation in genetic material. (Cells normally add methyl groups to DNA to turn off genes, during development and throughout life.) Mitalipov and Laurent found that, compared with iPS cells, the SCNT cells have methylation profiles of nuclear genes that are more ES cell-like. And the SCNT cells better maintained those patterns, unlike iPS cells, which started with and further racked up methylation “abnormalities” as time went on, Mitalipov says. “The bottom line is that the SCNT cells are more stable,” Laurent says. “And they are more similar to the IVF [ES cell] lines.”

Yet, stem cell researcher Marius Wernig of the Stanford University School of Medicine in Palo Alto, California, cautions that the differences seen so far between SCNT and iPS cells might simply reflect the normal variation seen within different ES cell lines and from iPS cell to iPS cell, some even derived from the same donor. It is too early to “draw any strong conclusion. It requires more than one lab to do these assays,” he says.

The work “settles a long-standing question: whether nuclear transfer works in humans,” says Rudolf Jaenisch of the Whitehead Institute for Biomedical Research in Cambridge, Massachusetts. “Whether these cells are different than iPS, that has not yet been resolved.”

Mitalipov concedes that the data suggesting the cell types differ is “very preliminary” and acknowledges that using nuclear transfer to produce ES cells is far more costly and more tightly regulated than creating iPS cells. However, he says that he still hopes “that the few labs that do have this expertise and hopefully some other colleagues will create a large bank of cloned ES cells from different disease patients. That is actually our goal: to provide this resource.”

If that resource is meant to help answer questions about iPS cells, such as their capacity to give rise to all cell types, Jaenisch is skeptical. Straightforward ES cells would better serve that role, he argues. Creating SCNT cells is “difficult” and it is a “hassle to get approvals,” he says. “Is it necessary? I am not sure.”

—TRISHA GURA

Trisha Gura is a science writer in Boston.

CREDIT: ISSCR

U.S. EDUCATION

Science Standards Begin Long, Hard Road to Classroom

Matthew Krehbiel's persistence has paid off. Krehbiel oversees science education for the Kansas department of education, and for the past 2 years he has briefed his state's Board of Education every month on how the department is getting ready to join a national initiative to redefine the teaching of science in U.S. elementary and secondary schools.

Last week, the board voted 8 to 2 to adopt the Next Generation Science Standards (NGSS). The action makes Kansas the third state (after Rhode Island and Kentucky) to embrace the voluntary standards since they were rolled out in April by a consortium of 26 states and a slew of private organizations, including AAAS (which publishes *Science*) and the National Academies' National Research Council.

A former high school science teacher, Krehbiel is gratified to see his hard work pay off. But he and other leaders in the NGSS movement know that implementation could take many years. The nascent debate over NGSS also promises to shine a spotlight on science, a subject that has traditionally played second fiddle in the public's eye to mathematics and reading. Science educators are both enthused and anxious about the chance to argue their case on a national stage.

"It's relatively easy to achieve the first goal of adoption [of NGSS]. But the scale of 'Now what?' remains immense," says David Evans, executive director of the National Science Teachers Association (NSTA).

A reminder of the rough road ahead came 2 days after the Kansas vote, when a Washington, D.C., education think tank gave NGSS a "C" grade. In a 66-page report by a panel of seven scientists, the Thomas B. Fordham Institute argues that NGSS is weaker than existing science standards in as many as 20 states.

"Yes, there will be real pushback in some states. So we're encouraging everyone to be patient," says Stephen Pruitt, head of Achieve Inc., a nonprofit in Washington, D.C., formed by state governors and industry that is coordinating NGSS-related activities (nextgenscience.org). "We think [widespread implementation] will take 3 to 4 years."

In development since 2009, NGSS is following a path plowed by the Common Core State Standards Initiative, which covers reading and mathematics. Both initiatives require states to develop and implement a new curriculum with relevant classroom materials, train both veteran teachers and those preparing to enter the field, and draw up tests that align with the standards. To borrow an analogy from field sports that others have used, standards set the bar for students; the curriculum is how they train to clear it;

The Fordham Institute's C grade reflects a fear that students will spend too little time learning the facts and, thus, won't have enough knowledge to apply. Its report also says that the standards are not sufficiently rigorous to prepare the country's more than 50 million precollege students for college-level work and jobs and that they slight the mathematics needed in high school physics and chemistry courses.

Pruitt argues that the Fordham analysis is outdated: "They are using the last-generation standards to judge the next-generation standards we have developed." Addressing the National Academies' Board on Science Education (BOSE) a day before Fordham released its critique, Pruitt said that "we need to redefine rigor. As it's now used, rigor has led to a culture of rigor mortis." Margaret Honey, a member of BOSE and president of the New York Hall of Science, agrees with Pruitt and thinks that it is important "to combine rigor with curiosity and engagement."

Critics of NGSS often equate rigor with a fine-grained treatment of a topic, Pruitt says. But NGSS consciously avoids what he calls "worksheet standards"—skills that a student can demonstrate simply by doing a set of homework problems. Fordham's criticism that NGSS calls for too much "practice" and too little "content" reflects a fundamental misunderstanding of the two terms, Pruitt adds. "Practice requires students to know the content so that they can then seek out the appropriate evidence," he says.

Politics aside, Evans says that NSTA is scrambling to help not just its 60,000 members get ready for NGSS, but also to provide the necessary support to the nation's 3.2 million teachers of K–12 science. "More than 80% of the teachers we surveyed are excited about NGSS and think it will make an enormous difference," he says (see graphic). "But barely one-third expect there will be sufficient resources to help them do what they think will be necessary. Resetting large numbers of teachers and giving them what they need is a really daunting challenge."

—JEFFREY MERVIS

While **83%** of teachers say NGSS will improve student learning ...

... 38%
think they will receive the additional training needed

... 42%
think their district will give them the appropriate classroom materials

... 41%
think they will have access to the necessary equipment

Help wanted. In a recent online survey, most NSTA members said they like the Next Generation Science Standards but worry about how it will be implemented.

and test scores measure their performance.

But NGSS expects to take a more leisurely pace to implementation than the Common Core, which has drawn the ire of groups who consider it a federal intrusion on local education matters. Some 30 states adopted the Common Core within its first 6 months, and by next year all consortia members—45 states and the District of Columbia—have promised to begin testing students on material aligned to the new standards.

One major difference between NGSS and current science standards is that NGSS tries to simultaneously get students to understand how science is done (practice) and its practical value (applications) along with mastering content (facts). Too often, NGSS proponents say, students are now asked to simply memorize definitions and equations and parrot back that knowledge on a test.

"As one Kansas school board member said before the vote," Pruitt recalls, "There's no way we can know everything in science anymore. But we should know enough to understand anything new and apply anything old." I think that's just brilliant."

NUCLEAR NONPROLIFERATION

Iranian 'Medical' Reactor Stokes Search for Alternatives

If all goes to plan, Iran next year will switch on a facility that gives nuclear nonproliferation analysts goose bumps: the Arak heavy water reactor in the central province of Markazi. Iranian officials have long stated that a chief aim of the fission reactor, known as the IR-40, is to make radioisotopes for medicine. But it also will yield something far more troubling: about 10 kilograms of plutonium a year, enough for one or two atomic bombs.

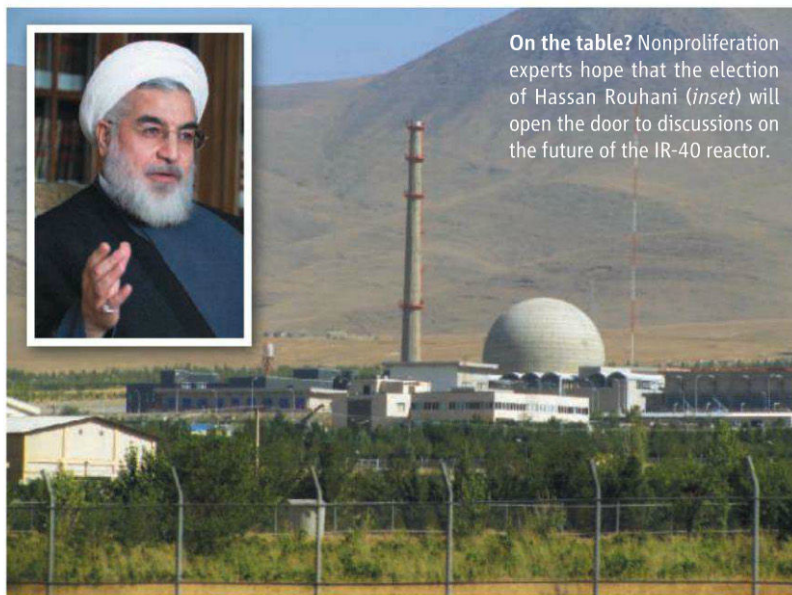
Locked in a standoff with the United States and allies over its nuclear ambitions, Iran has steadfastly averred that the objectives of its sprawling nuclear program are peaceful: to generate electricity and produce radioisotopes for industry and medicine. But what if Iran didn't need the controversial IR-40 to make medical isotopes? A new report by nuclear specialists highlights that possibility, laying out alternatives that avoid uranium and production of plutonium, the fissile material in nuclear bombs.

Medical isotopes are a ripe topic for diplomacy. In remarks on 17 June reported by Fars News Agency, Iran's President-elect, moderate cleric Hassan Rouhani, said that his nation is prepared to "increase transparency" of its nuclear program and "enhance mutual confidence [building] between Iran and other countries." One confidence-building measure, diplomats say, might be expanding medical isotope production via ways that don't facilitate making bombs. If U.S. negotiators "can sell the idea of Iran participating in advanced nuclear technologies [that steer clear of fissile material], then maybe you've got something," says Mark Jansson, special projects director at the Federation of American Scientists in Washington, D.C.

Radioisotopes are widely used in medical imaging and cancer treatment. Considered a dividend of nuclear technology, they were an important reason that nuclear powers in the 1950s and 1960s promoted the construction of research reactors around the world. However, many medical isotopes are made

in reactors that use highly enriched uranium (HEU), which contains at least 20% of the fissile isotope uranium-235 (U-235)—enough to be "weapons ready." So arms control advocates are loath to see HEU-powered reactors spread, and have successfully shut down some located in unstable regions.

In Iran, technicians already make medical isotopes in an aging reactor that uses uranium enriched to 19.75% U-235—a hair below bomb-grade. The IR-40 would replace that reactor, but use natural uranium, which is mostly U-238, and not HEU. That worries arms control specialists because bombarding natural uranium with neutrons turns out to be a very efficient way to generate plutonium.



On the table? Nonproliferation experts hope that the election of Hassan Rouhani (inset) will open the door to discussions on the future of the IR-40 reactor.

Several alternative methods of generating desired medical isotopes would make it harder for would-be proliferators to lay hands on weapon-grade fissile material, argues the 13 June report from the Center for Science, Technology, and Security Policy of AAAS (publisher of *Science*). The technical approaches include an expanded reliance on cyclotrons or spallation neutron sources. High demand for short-lived isotopes used in positron emission tomography, coupled with technical advances in miniaturizing accelerators, has driven down costs. "Accelerator technology is far less expensive and more capable than in the past," write authors Derek Updegraff and Seth A. Hoedl, analysts at AAAS.

Their report focuses on the world's most widely used medical isotope: technetium-

99m (Tc-99m), which is employed in about 80% of all nuclear medicine procedures—some 30 million a year. Tc-99m is popular because it readily concentrates in various tissues for imaging. Hospitals that use Tc-99m for single-photon emission computed tomography, or SPECT, purchase its parent radioisotope, molybdenum-99 (Mo-99), which is a decay product of U-235. The main way to produce Mo-99 now is to irradiate enriched-uranium targets in a research reactor. But it doesn't have to be that way: Mo-99 can be created by a photonuclear reaction on Mo-100, or Tc-99m can be created directly by bombarding Mo-100 with protons. The latter approach, the report notes, is now

being tested by a Canadian cyclotron maker, which this month announced that hospital accelerators can be adjusted to produce Tc-99m. "The proliferation risk with accelerators is dramatically lower," Updegraff says. Their report also analyzes all the other most commonly used radioisotopes for diagnostics or therapy and finds that in principle, all these isotopes can be created without a nuclear reactor.

If nonproliferation is the objective, cost may not be a showstopper. In the wake of last week's Iranian elections, U.S. officials hope to revive nuclear

talks, which broke down in April. Rouhani, a former nuclear negotiator in Iran's talks with the West, has not signaled any major policy shift. And the window for a deal on IR-40 may be closing. After Iran has spent 10 years planning the facility, "it will be hard to convince them to walk away" from the reactor, Jansson predicts. But proliferation risks could be reduced, he notes, by fueling it with low-enriched uranium, which would yield less plutonium.

Whatever happens with IR-40, the long-term implications of alternative isotope technology are broader than Iran, says Pierce Corden, a disarmament expert and visiting scholar at AAAS who initiated work on the report. "There may be other problematic situations in the future," he says.

—RICHARD STONE

CREDIT: NANKING2012/WIKIMEDIA COMMONS; (INSET) MOJTABA SALIMI/WIKIMEDIA COMMONS



HIGH-ENERGY ASTROPHYSICS

Global Gamma Observatory Looks for (Two) Homes



Flash mob. The CTA will look for flashes from the upper atmosphere caused by cosmic rays from deep space.

global weather models. The CTA Consortium will also consider construction costs, communications, and how much the home country is willing to spend on infrastructure and equipment. Once sites are chosen, the delicate negotiations over each country's level of funding will begin.

"We are facing a 'What's first, the chicken or the egg?' kind of situation as funding agencies

An ambitious project to build twin observatories capable of detecting gamma rays—high-energy photons—from deep space has reached a crucial phase as the consortium of 27 countries must decide which two of nine possible sites will host the Cherenkov Telescope Array (CTA). The nations vying to provide homes for the €200 million instruments must file reports on the seeing conditions at their sites by the end of this month, and the CTA Consortium will then make a choice by December. "Right now everything is so uncertain that anyone could win," says Alberto Etchegoyen, director of Argentina's Institute of Technology in Detection and Astroparticles in Buenos Aires, who presented his country's site during a CTA meeting last month in Chicago.

CTA will be the largest gamma ray-hunting instrument ever constructed. Its two observatories will be 10 times as powerful as current instruments and will seek to answer two key questions: where do cosmic rays come from, and what is the elusive dark matter that makes up 85% of all the matter in the universe. "It will also show us unknown unknowns, totally new phenomena we've never seen before," says astrophysicist Rene Ong of the University of California, Los Angeles, one of 1000 CTA Consortium members. Six nations are in the race—the United States, Mexico, and Spain are bidding for the northern site, and Argentina, Chile, and Namibia for the southern—with some offering more than one location.

Gamma rays are the most energetic form of electromagnetic radiation and come from violent cosmic locales such as supernova explosions, neutron stars, and black holes. Detecting gamma rays from the ground is hard because they collide with air particles in the upper atmosphere. But the collisions also trigger cascades of secondary particles, some of which start out traveling faster than the speed of light in air and emit flashes of

blue light known as Cherenkov radiation as they decelerate.

The first prototype Cherenkov telescope, built from a trash can with a searchlight mirror and a photon detector mounted inside, was assembled in the 1950s in the United Kingdom. Today's largest Cherenkov telescope is the HESS telescope in Namibia, which has five mirrors, the largest of which is 28 meters across (*Science*, 3 September 2004, p. 1393). Because they are not looking out into space, Cherenkov telescopes do not need the high-precision mirrors or mountaintop locations of normal astronomical telescopes. Instead, they need a large collecting area and clever electronics to pick out the faint Cherenkov flashes from the background noise. From those signals, astronomers can infer the energy of each incoming gamma ray and the rough direction of its source.

The CTA will be the first global-scale instrument, able to view the whole sky and cover an unprecedented range of gamma ray energies from about 20 gigaelectron volts to more than 100 teraelectron volts, so it will catch previously undetected particles. To do so will require some 120 telescopes: 20 spread over 1 square kilometer at the northern array and 100 distributed over 10 square kilometers at the southern site.

"This is the ultimate experiment. Nobody will build a larger telescope of this kind ever," says Trevor Weekes of the Whipple Observatory in Amado, Arizona, who pioneered Cherenkov telescopes in the United States. "The scientific return is 100% guaranteed," says Jamie Holder, spokesperson for Whipple's VERITAS telescope. "Only around 10% of the sky has been observed with reasonable sensitivity so far," he says.

The key site criteria are a large number of cloudless nights, low humidity, and low wind. Researchers have had weather instruments at all the sites for at least a year and have studied local weather reports, satellite data, and

don't want to promise any amount until a site is chosen, and the consortium is eager to know how much each country will contribute so they can make a better decision," says William Lee, a physicist at the National Autonomous University of Mexico in Mexico City. Spain has proposed a site on the island of Tenerife but is silent about money. "The Ministry of Economy supports us but has not yet committed any funding," says Ramón García López of the Astrophysics Institute of the Canary Islands. And Chile, although it has a candidate site put forward by local CTA scientists, is not yet a member of the consortium.

But there are also many positive signs. Argentina has "committed to spending €12 million on infrastructure and scientific instrumentation and, if we are selected, our project will also be supported by Brazil," Etchegoyen says. CTA is included in the European Union's road map of scientific infrastructures and is one of seven top-priority particle physics experiments according to Europe's funding agencies. The U.S. National Academy of Sciences backed the project in its 2010 decadal survey of astronomy. And in April, Germany delivered the first medium-sized telescope prototype and pledged €50 million for construction.

Once the site reports are all in at the end of this month, an external committee will analyze them and a final site recommendation will be issued in December by the CTA Resource Board, representing "around 15 funding agencies from the project's main contributing countries," says Manel Martínez, CTA co-spokesperson. "I'm quite optimistic we will get approval [for construction] by the end of 2014," says Werner Hofmann, CTA spokesperson. CTA could then deliver its first scientific results in 2017 and is expected to be fully operational by the end of 2019, he says.

—NUNO DOMINGUEZ

Nuno Dominguez is a writer in Madrid. With reporting by Daniel Clery.

CREDIT: G. PÉREZ/AC/SM/THE CTA CONSORTIUM; (INSET) DESY



How Long Can the U.S. Stay on Top?

Government funding has powered U.S. research universities to global preeminence. With that support now shaky, can philanthropy help them maintain their lead?

JOHNS HOPKINS UNIVERSITY BEARS THE NAME OF THE MARYLAND banker who in 1873 made what was then the largest bequest in U.S. history, some \$7 million, to create a research university and hospital. Today, the name of another record-setting philanthropist is nearly as ubiquitous on the Baltimore campus.

The Bloomberg Center for Physics and Astronomy, the Bloomberg School of Public Health, and the Bloomberg Children's Center hospital are prominent reminders of the \$1.1 billion donated over the past 30 years by Michael Bloomberg, the billionaire media mogul and outgoing New York City mayor. And his generosity goes well beyond bricks and mortar: In January, Bloomberg (Hopkins class of 1964) announced that he was donating \$250 million to endow 50 new faculty chairs and \$100 million to provide scholarships to needy undergraduates.

Such gifts aren't just eye-catching: In an era of stagnating U.S. government support for academic science, officials at Hopkins and other top research universities in the United States say that private philanthropy is increasingly essential in maintaining their elite status. In one popular ranking of the world's best universities, by Shanghai Jiao Tong University, U.S. institutions now hold 19 of the top 25 slots.

But many of those top universities worry that their ratings could slip if the largest single slice of their revenue pie, funding from the federal government, starts to shrink. Their anxiety is heightened by

the increased spending on academic science elsewhere in the world, notably China and other Asian nations.

To stay on top, many schools are aggressively pursuing a time-tested American strategy for fueling growth: asking affluent donors for money. Despite the recent global recession, that strategy is paying off handsomely. Last year, for example, Stanford University became the first nonprofit institution in the world to raise more than \$1 billion in private donations in a single calendar year.

Online

sciencemag.org

Podcast interview
with author Jeffrey
Mervis (http://scim.ag/pod_6139).

Private institutions are by no means the only ones cashing in. Driven by declining support from their states, public institutions have also become aggressive fundraisers.

"We are much more reliant on philanthropy than ever before," says Robert Birgeneau, outgoing chancellor of the University of California, Berkeley, the state's flagship university. "I think we've survived the crisis and we've transitioned to a new model."

This story is the last in *Science's* series on what it takes to be a top global research university. It examines threats to America's academic research prowess. Most university presidents believe that the new model is sufficient to keep their institutions on top. But some worry that countervailing trends could defeat that strategy.

CREDIT: JAMES T. VANRENSELAER/HOMEWOODPHOTO.JHU.EDU

A big thank you. Michael Bloomberg, who has donated \$1.1 billion to Johns Hopkins University, was awarded an honorary degree in 2003.



A rapid rise

Although the average American may think that the United States has always led the world in academic science, its preeminence is a relatively recent phenomenon. To be sure, top-rated universities such as Harvard, Princeton, and Yale have been around for centuries. But others on the list, such as Hopkins and Stanford, date from only the late 19th century. And, as a nation, the United States didn't become the unquestioned leader in university-based research until the past half-century.

"The center of intellectual excellence has moved very often over the centuries," says biologist Shirley Tilghman, who is stepping down this month after 12 years as president of Princeton University. "It started with Bologna and the great Italian universities. It moved to Germany, then to England, and only relatively recently across the Atlantic to the United States. That suggests that the center of gravity could easily move outside the United States if we stop paying attention to the ingredients that make our great universities the magnets they are today."

Drew Faust, president of Harvard University, says that it's a mistake to think that the great schools rise and fall on the

basis of some immutable historical clock. "There's something fatalistic and deterministic in saying that this is cyclical and we should just sit back and say that our cycle is done," says Faust, a historian. "The United States is still the strongest economy in the world and has the strongest university system. Whether one or both of those things persists depends on us, not on some predetermined fate."

The top U.S. schools have achieved and maintained excellence through a combination of historical, economic, and political factors, says Princeton historian Anthony Grafton. Historically, "a really powerful national commitment to research" helped create great institutions, says Grafton, citing Germany and Great Britain as nations that saw the geopolitical advantages of funding science. "But that's not enough. Without World War II and Sputnik, [the United States would] really be nowhere. Those events created a feeling that our national security rested on a commitment to research."

That argument was crystallized by engineer and White House adviser Vannevar Bush in *Science: The Endless Frontier*. His influen-

tial 1945 report, requested by President Franklin Roosevelt, emphasized the importance of federal support for academic research. "The approach required pumping in money across the board, rather than to the islands of excellence that already existed," Grafton explains. "Research became what the university is about." The report led to the creation of the National Science Foundation (NSF) and fueled the rapid expansion of the National Institutes of Health (NIH).

Publicly financed tuition allowed millions of World War II veterans to go to college on the GI Bill in the first decade after the war. But once that wave subsided in the early 1950s, he says, institutions began to contract. It was actually their children, the so-called baby boomers, who provided the demographic push that launched today's academic research juggernaut. But a new set of demographic and economic trends are prompting U.S. research universities to once again rethink their strategies.

Financing growth

A look at how elite U.S. universities are funded explains why philanthropy is becoming so important to the bottom line. Just as a great white shark must keep moving through the water to extract enough oxygen to avoid drowning, a top-ranked research university must ingest ever-increasing amounts of money to pay for the new buildings, updated equipment and facilities, promising students, and world-class researchers required to stay on top. That imperative largely explains why, in recent decades, Stanford, Harvard, the Massachusetts Institute of Technology, the University of Michigan, and other research powerhouses have grown at a rate that far outpaced the overall economy.

Even as U.S. personal incomes stagnated over the past dozen years, the budgets of elite U.S. universities roughly doubled. And that increase has generally been poured into research rather than into creating a larger pool of undergraduates. While the overall budget of Stanford University, for example, has grown from \$1.8 billion in 2000 to \$4.4 billion this year, the size of its freshman class has remained at about 1750.

Parents paying ever-rising tuition costs might think that they have been fueling that growth. But they would be wrong. Even at an elite private school like Stanford, with an undergraduate sticker price of \$60,000 a year, student income is providing only 17% of this year's overall revenues. And with the public and politicians questioning the value of an education and demanding greater accountability, raising tuition by more than a small percentage each year has become a risky proposition.

Other revenue sources are also under siege. Car-



GLOBAL RESEARCH UNIVERSITIES

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This is the last of six stories about creating and maintaining global research universities. Previous stories have examined the role of mobility (7 September 2012, p. 1162), satellite laboratories (28 September 2012, p. 1600), European efforts to strengthen elite universities (2 November 2012, p. 596), the problems at a Saudi Arabian startup (7 December 2012, p. 1276), and how to make academic research pay off (15 February, p. 750).



"We need to have a conversation between the government and the research universities on how to live at steady state."

—SHIRLEY TILGHMAN,
PRINCETON UNIVERSITY

CREDITS (LEFT TO RIGHT): DENISE APPLEWHITE/PRINCETON UNIVERSITY; OFFICE OF COMMUNICATIONS; ISTOCKPHOTO

ing for patients provides a significant income for universities with medical schools and hospitals. But efforts to rein in the cost of health care, including provisions in the 2010 law known as Obamacare, are putting the squeeze on academic medical centers.

At public universities, state funding historically paid a large portion of the bills. But that source has steadily declined over the past 2 decades. Many flagship research universities now describe themselves as “state-assisted” or even “state-allied” to reflect the diminished role of government.

“When I joined the law school faculty 35 years ago, the state appropriation made up about a third of our budget,” says Mark Nordenberg, who has been chancellor of the University of Pittsburgh in Pennsylvania since 1995. “It’s now well under 10%. If the board of trustees had been judging me on that measure alone, they would have found a new chancellor a long time ago.”

At elite research universities, the two biggest revenue streams are typically sponsored research (overwhelmingly from the federal government) and investment income (meaning philanthropy and the income generated from a school’s endowment). Traditionally, however, research dollars are more prestigious because they are awarded through competitive peer review by funding agencies such as NSF and NIH. Those dollars are so important that federal funding has become a *de facto* measure of overall quality for a university.

Nordenberg, for example, notes with considerable pride that his institution ranks fifth on a list of U.S. universities receiving NIH research dollars. “Penn [University of Pennsylvania] is fourth, and we’re going to catch them,” he predicts. Thanks to its renowned medical center, Pittsburgh also ranks 10th in overall federal research funding.

At Vanderbilt University in Nashville, Chancellor Nicholas Zeppos touts the fact that Vanderbilt’s research portfolio has grown five times faster over the past decade than the budget of NIH, its primary source of research funding. To Zeppos, that rate of growth helps explain a parallel rise in the university’s performance on national surveys of overall excellence.

Zeppos also points to the fact that Vanderbilt ranks among the top 10 of more than 100 U.S. medical schools in the amount of federal funding received per square foot of space devoted to research. That metric, he says, means the university provides the government with a bigger bang for its buck than most institutions—an important selling point in today’s fiscal environment.

Such efficiency requires considerable attention to detail, he admits. But Zeppos, a former lawyer and law professor, says he sees no problem applying such management practices to an enterprise that has historically resisted calls for greater accountability. “Remember, as a lawyer I used to have to account for my time in 6-minute increments,” he notes.



“The United States is still the strongest economy in the world and has the strongest university system.”

—DREW FAUST,
HARVARD UNIVERSITY

Philanthropy to the rescue?

Uncertainty over the future of major revenue streams is helping supercharge interest in philanthropy at major research universities. For some, it’s a return to their roots: Bequests from wealthy individuals created and sustained many of today’s elite U.S. research universities, which, in turn, adopted the family names of their donors. Hopkins was on the leading edge of a cohort of industrial barons turned philanthropists who include Cornelius Vanderbilt, Ezra Cornell, Leonard Case Jr., Andrew Carnegie, and Richard Mellon, all now memorialized on sprawling campuses bearing their names.

It’s hard to overstate the historical importance of academic giving, says Subra Suresh, who this spring stepped down as NSF director to become president of Carnegie Mellon University in Pittsburgh. “In 1900, Andrew Carnegie gave \$1 million to the city of Pittsburgh and then Andrew Mellon created the Mellon Institute of Science. And in 1967 they merged,” says Suresh, who starts his job on 1 July. “So Carnegie Mellon came into existence as a result of philanthropy, as did lots of other institutions.”

Such stories reflect a uniquely American tradition that, fostered by tax and estate policies, continues today. Last year, for example, academic giving totaled \$31 billion, according to the Council for Aid to Education (CAE), a New York-based nonprofit that tracks private giving to education. The amount is just \$600 million below the all-time high set in 2008 before the global financial crunch hit home.

For 29 of the past 30 years, either Harvard or Stanford has been ranked No. 1 on the list of top fundraising universities, according to CAE. And it’s probably no coincidence that they also rank first and second, respectively, in the Shanghai rankings.

“Philanthropy is hugely important to us,” says Harvard’s Faust. “Our endowment generates about 35% of our operating budget. And it’s the result of centuries of philanthropy.”

At Stanford, investment income (donations and the interest from its endowment) this year generated \$1.1 billion, one-quarter of the school’s \$4.4 billion operating budget. That amount almost matches the \$1.27 billion in research grants that Stanford’s faculty attracted. But the two funding streams are trending in opposite directions, with donations gaining momentum and research grants likely to be squeezed by the tightening federal budget.

Stanford’s billion-dollar fundraising year, for instance, came shortly after the university concluded a 5-year capital campaign, labeled The Stanford Challenge, which raised \$6.2 billion. That total exceeded Stanford’s goal by nearly \$2 billion and set a record for any university. But the record may not last long. This fall, Harvard will launch a capital campaign that, according to rumors, could aim to raise more than \$7 billion.

Private angels have helped the University of California, Berkeley, offset reduced state government support. State funding as a percentage of the university’s overall budget has dropped from 30% to 11% since 2004. “If philanthropy disappeared, that would have a huge negative impact on us,” Chancellor Birgeneau says.

Generous donors may be even more important to Cornell NYC Tech, a startup university that hopes to spur innovation in and around New York City. A \$350 million donation from alumnus Charles Feeney helped Cornell win a competition to establish the school, and Cornell officials are counting on philanthropy to generate most of the \$2 billion needed to build a modern campus on the city’s Roosevelt Island.

This spring Irwin Jacobs, the founder of Qualcomm, and his wife, Joan, gave \$133 million toward the largest component of the new university, the Technion-Cornell Innovation Institute. The gift earned the couple naming rights and was tangible evidence that

Grateful Patients Can Lead To Gracious Gifts

For most alumni, university fundraising may seem to be uncoordinated and lacking in focus—an assortment of phone calls, solicitous letters, and invitations to a class reunion. But for Steven Rum, it's a science. And the goal is to carry out more research.

Rum is senior vice president for development and chief fundraiser for Johns Hopkins Medicine in Baltimore, Maryland. Last year, his team had a banner year, raising \$318 million. Their approach places the physician scientists at Hopkins on the donor front lines. The goal is to turn the positive feelings of "grateful patients" into support for new research, faculty chairs, academic scholarships, bricks and mortar, or simply defraying the cost of running a multibillion-dollar medical center.

"What most appeals to donors is research," says Rum, a former professional baseball player who has spent 25 years raising money for non-profit organizations. "They want to be involved in changing the way we treat or eliminate disease. Most donors don't want to see their names



High touch. Johns Hopkins's Harvey Singer shares his research on pediatric neurological diseases with parents.

on a building. They think the institution should be responsible for the physical plant. They want to support people."

Rum has 65 full-time fundraisers on a staff of 165. Each one is responsible for meeting weekly with physicians—their "caseloads" range from a dozen to more than 30 docs—to discuss which of their patients might be potential donors. The conversation is designed to help them identify what Rum calls a donor's "qualifying interest" and connect it to their "capacity," that is, the ability to make a donation.

More often than not, Rum's team finds that

sweet spot. Despite generating an impressive annual yield of \$225 million—last year was a record—Rum thinks that universities could do even better if they tried harder. "Charitable giving has been at 2% of GDP for years," he says. "And the reason that figure has been flat is the lack of investment on the development side."

Rum says studies have found that a well-run development office can yield a 10-fold return on the university's investment, that is, on salaries and benefits. And he thinks that figure is probably low.

"Ideally, I'd like to have one gift officer manage no more than six doctors," he says. "If managed properly, that office would generate a list of 125 qualifying names a year. By the end of the first year they would have paid for themselves, and by year 4 or 5, when they've hit their stride, they would be raising 4 or 5 million a year."

However, achieving that level of payoff requires a long-term commitment to fundraising that not all universities are willing to make, he says. "It takes years to develop this culture. And if a doctor is offended by talking with a development officer, or by discussing money with a patient, then you won't be successful." —JDM

the institute's vision could be realized, says Craig Gotsman, who directs the new institute.

A computer scientist and serial entrepreneur at Israel's Technion, Gotsman believes that the institute's approach to training high-tech workers and researchers with an entrepreneurial bent matches Mayor Bloomberg's vision for a new type of graduate science university. But its future was not at all assured when Cornell won the competition

"There are several large foundations in our area that see a link between strong universities and a vibrant regional economy, and they have made support for universities a high priority," Pittsburgh's Nordenberg says. He cites a 1984 grant from the Richard King Mellon Foundation that established the University of Pittsburgh Cancer Institute, which has become an NIH-funded comprehensive cancer center.

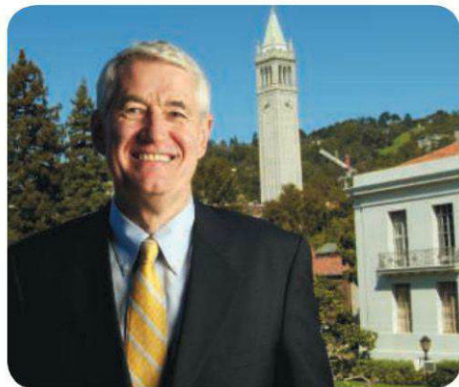
Amid such philanthropy successes, there are hints of concern. Nordenberg, for instance, worries that declining government support could make it harder for universities to keep winning major foundation grants. "Foundations have looked to the federal government to leverage their investments," he says, "and I'm not sure they would make the same investments if they didn't believe the federal government was going to do its part."

Princeton historian Grafton sees a deeper problem. He thinks that university presidents are taking a big gamble by counting on philanthropy as a long-term solution to fueling growth, especially with the actual cost of an education lagging behind what students pay. "The top schools, especially the privates, have this very precarious structure," he says, "which basically rests on the assumption that we subsidize everybody now, and then we get the money back 40 years later when they're rich. And nobody knows if that's right."

What if growth stops?

Ultimately, such discussions come down to a fundamental question: Can U.S. research universities retain their preeminence if their public and private funding flattens out?

A no-growth scenario would be traumatic, several presidents told



"I think we've survived the crisis and we've transitioned to a new model."

—ROBERT BIRGENEAU,
UNIVERSITY OF CALIFORNIA,
BERKELEY

over a Stanford-led team in December 2011.

"At that point we were living on love and fresh air," Gotsman confesses. But "we never really considered having to close up shop." Acquiring a dependable and substantial revenue stream through philanthropy was a given, he says. And Jacobs's gift is not sufficient, he acknowledges. "We're not out of the woods."

Although huge individual gifts may garner the most publicity, institutional fundraisers spend most of their time cultivating smaller donors (see sidebar, above). Community-based foundations can also play a vital role.

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Science. “There are very important new areas of exploration into which we need to move,” Harvard’s Faust explains. “And if that occurs without growth, then there are important things we’re doing that we’d need to stop doing. We would need to make tradeoffs to free up resources for areas where there are expanding opportunities. And those tradeoffs will be very costly in terms of our research and education missions.”

Nordenberg agrees. “Our continued growth, to a large extent, is dependent on philanthropy,” he says.

Princeton’s Tilghman would like to see her colleagues spend less time on plans to keep growing and more time contemplating a no-growth universe. “It’s very hard to go onto any research university campus, particularly one with an academic medical center, and not see a new building going up,” she says. “So what is the expectation for how research in that building is going to be funded?”

“If you ask any medical school dean, they will say that it will be paid from a combination of direct and indirect costs funded by the federal government,” she continues. “Given the constrained prospects for increases in federal funding for the next, who knows, 5 to 10 years, that dean has to believe that, in order to pay for the building, his or her institution is going to outcompete, to an even greater extent than they do now, every other academic institution.

“But this just can’t be true for every academic medical center,” she says. “It does not compute. I think we need to have a conversation between the government and the research universities on how to live at steady state. I don’t see that conversation happening right now.”

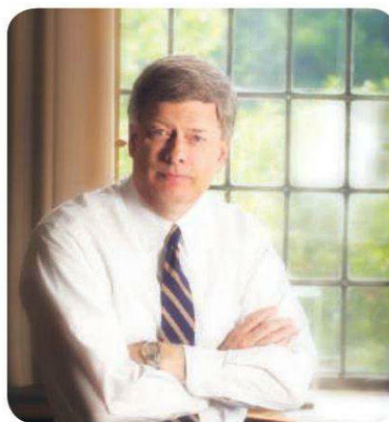
The assumption that their institution will be able to continue to indefinitely expand contributes to what Tilghman sees as an even bigger problem. “The greatest risk to American universities,” she says, “is complacency and self-satisfaction.”

The assumption that their institution will be able to continue to indefinitely expand contributes to what Tilghman sees as an even bigger problem. “The greatest risk to American universities,” she says, “is complacency and self-satisfaction.”

The global challenge

Despite her concern over growth, Tilghman joins her peers in offering an upbeat assessment of several other developments often seen as potential threats to U.S. preeminence in academic research. Some analysts predict the booming science budgets of China and other Asian countries, for instance, will make it harder for U.S. institutions to recruit the best and brightest. But California’s Birgeneau sees Asia’s academic growth as a welcome addition to the global research enterprise.

“We’re about creating knowledge, and the more people there are who create knowledge, the better,” he says. “Likewise, the more educated the world is, the better off civilization will be.”



“Our continued growth, to a large extent, is dependent on philanthropy.”

—MARK NORDENBERG,
UNIVERSITY OF PITTSBURGH

Having more world-class universities will increase the competition for faculty members, the presidents acknowledge. But elite U.S. universities are accustomed to holding bidding wars for the best researchers.

“I find it peculiar when people ask if I’m going to hire someone from another university,” Vanderbilt’s Zeppos says. “What big, ambitious institution doesn’t do that? And how do the best universities know they have the best talent unless someone is knocking on their door trying to recruit them away?”

The increased capacity in other countries has also made international collaboration even more attractive, several presidents say. “I’m really heartened by the type of intellectual partnerships that are happening all over the

world that allow us to fund really expensive science,” Tilghman says. The Large Hadron Collider at CERN outside Geneva, Switzerland, and the Atacama Large Millimeter/submillimeter Array telescope in Chile are two examples of several countries pooling resources to do research that none could afford on its own, she notes.

Closer to home, the dramatic growth in the past 2 years of massive open online courses, known as MOOCs, is a trend that every university president is watching. But none think that this new technology will undermine the quality of education on their campus, a development that could make it harder to attract talented students, especially from other countries.

“If you talk to those students, a big part of what they want to do is to be in America and learn from interacting with Americans,” Nordenberg says. “And you can’t do that effectively without being here. And when students look back at their experiences and say how much they learned, it’s often because of the interactions outside of the classroom, with a faculty member or other students, or through some extracurricular activity.”

Making the future

Such optimistic assessments may come naturally to university presidents, Grafton says. “If you’re asking people for money, you have to pretend that everything is fine,” he says. “Because if your institution looks like it’s in trouble, nobody will give you money.”

Birgeneau is taking such can-do thinking a step further: After he steps down from the top job at Berkeley, he’ll devote much of his time to raising funds for a \$10 billion initiative, called the Lincoln Project. The plan is to pool federal, state, and private money to create 10,000 new endowed chairs at U.S. research universities, he says. Reaching that goal “will require a redirection of funds,” he admits. But “when legislators tell me there’s no money, my answer is that there’s lots of money. It’s just a question of what we spend it on.”

Grafton says he wants to believe that is true. “Every fiber of my being hopes that this world can go on,” he says, and that U.S. research universities can sustain their heady success. “But it’s hard to extrapolate a happy future 20 years down the road.”

—JEFFREY MERVIS



“How do the best universities know they have the best talent unless someone is . . . trying to recruit them away?”

—NICHOLAS ZEPPOS,
VANDERBILT UNIVERSITY

CLIMATOLOGY

Monsoon Melee

The rhythms of life across South Asia depend on the Indian monsoon. Climate scientists are locking horns over the cause of the summer deluges

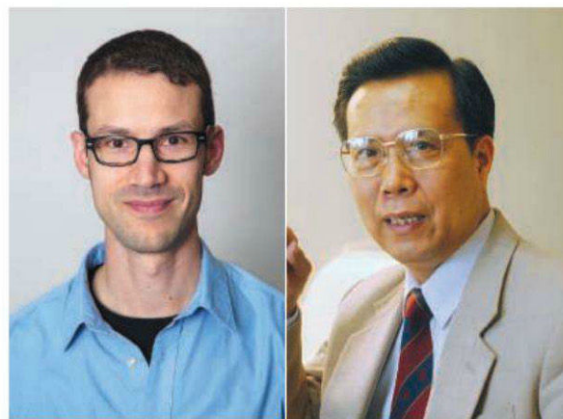
BEIJING—The Tibetan Plateau is a study in extremes. It's the world's largest expanse of elevated terrain, covering 2.5 million square kilometers. Frigid in winter, it bakes in summertime: Because the average altitude tops 4000 meters, the vast landscape absorbs far more solar energy than land at sea level. As a result, models predict that in summer, the air layer above the plateau can be much warmer than air at a similar altitude over the oceans or over land lying at sea level.

It's like "having a gigantic heat pump at over 4000 meters," says Wu Guoxiong, an atmospheric scientist at the Chinese Academy of Sciences' Institute of Atmospheric Physics here. The temperature differential is the engine that drives the spectacular summer deluges in South Asia known as the Indian monsoon—or so say textbooks. A new spate of research is challenging existing dogma, sparking a debate over the Tibetan Plateau's role in the Indian monsoon. How it's decided could have effects reaching far beyond the climate science community.

At stake are how to "predict when [the monsoon] will start, how long it's going to last, and how it will respond to rising carbon dioxide and aerosols," says David Battisti, a climate scientist at the University of Washington, Seattle. "That matters hugely to millions of people." A resolution could prove elusive, however, because of the challenges of modeling climate in mountainous regions.

This much is agreed upon: In the summer, the air over and around Tibet is much

warmer than air at the same altitude over the Indian Ocean. Consensus breaks down on whether that heating effect has broad consequences. In the 1950s, atmospheric scientists Ye Duzheng and Hermann Flohn independently proposed that the temperature differential creates winds that blow moist air from the sea into the interior of the Indian subcontinent. There, the air warms and rises, creating monsoonal rains. The hypothesis, which has stood for decades, is supported by climate modeling: The mon-



Dueling theorists. Wu (right) says the "heat pump" of the Tibetan Plateau drives the Indian monsoon. Boos (left) disagrees.

soon's strength—the total rainfall on the Indian subcontinent, that is—and how far north it reaches are greatly reduced when Tibet and the Himalayas are removed from climate models.

But those modeling studies have got it wrong, argues William Boos, a climate scientist at Yale University. They fail to "distinguish the role of the Himalayas from that

Peaks of contention. Modeling climate in the Himalayas and other mountainous regions is notoriously difficult.

of the Tibetan Plateau," he says. In 2008, he and Zhiming Kuang, then both at Harvard University, after examining temperature and humidity records in India and Tibet, uncovered what they claim are inconsistencies in the monsoon paradigm. One is that the upper atmosphere in the region is hottest over northern India and along the Himalayan ridge—not over Tibet. The finding "contradicts the view that Tibet is the heat center," says Peter Molnar, a geoscientist at the University of Colorado, Boulder. Secondly, Boos and Kuang showed that this hot air blankets the land surface with the highest energy level—a calculation based on temperature and the energy released by water vapor when it rises and condenses into liquid. That means heating of the Indo-Gangetic Plain, rather than Tibet, is driving the monsoon, Boos argues.

Supporters of a diminished role for the Tibetan Plateau also point to precipitation models, which hold that the hotter the surface is and the more moisture it contains, the more likely it is that water vapor will rise. Heat is released as water vapor condenses and rain falls, thereby transferring the energy of air near the surface to the upper atmosphere. More water vapor means a warmer upper atmosphere—exactly what Boos and Kuang saw.

In a series of papers, the most recent of which appeared in February in *Scientific Reports*, the two scientists argue that Tibet is irrelevant to the Indian monsoon. Instead, they say the monsoon is caused by a "barrier effect": the Himalayas blocking cold, dry winds from the north. In a global climate model, the duo showed that the Himalayas alone could generate a monsoon pattern that is largely similar to that pattern predicted when the Tibetan Plateau is included. When the mountain ranges were absent, they ended up with much lower energy in the air over northern India and got a weaker monsoon.

To further support their hypothesis, the researchers tweaked the model such that the Tibetan Plateau reflected all incoming solar radiation back into space, without heating the atmosphere. This "effectively turned off the heating source of Tibet without changing the Himalayas' blocking effect," Boos says. The result: The monsoon pattern hardly budged.

CREDITS (TOP TO BOTTOM): FENG WEI PHOTOGRAPHY/GETTY; TONY FIORINI; COURTESY OF WU GUOXIONG

Climate counterpoint

The new take on Tibet has shaken the foundation of how climate scientists understand the Indian monsoon, Molnar says. But not everyone is convinced. Wu, for one, argues that the reduced monsoon rainfalls that Boos and Kuang saw in their model could result from heating of the Himalayan slopes rather than from a barrier effect. To test this, Wu and colleagues performed another simulation in which they removed Himalayan heating. They came up with much less rainfall in the northern branch of the Indian monsoon—the rain belt stretching from the Bay of Bengal into Bangladesh, northern India, Myanmar, Nepal, and northern Pakistan. In a May 2012 report in *Scientific Reports*, the researchers concluded that the southern branch of the monsoon, which covers southern India, is mainly driven by land-sea thermal differences, while heating of the Himalayas is necessary for drawing moisture further inland.

Others have leapt to the defense of the plateau heating hypothesis. Peter Webster, a climate scientist at the Georgia Institute of Technology in Atlanta, questions whether the upper atmosphere over northern India is hotter than that over Tibet: The weather data that Boos and Kuang relied on are flawed, he asserts, but an equally fundamental issue is that “a hot surface isn’t enough to give you a monsoon,” he says. “The most effective way to heat up the upper atmosphere is through large, elevated land.” The heat and moisture that give the air high energy levels over northern India, Webster says, come from the warm ocean near the equator. This depends on a big temperature difference between the equator and the elevated terrain in a midlatitude swath of Asia, Tibet included, which sets up the winds that blow inland. The high energy of air over northern India “is a result, rather than the cause, of the monsoon,” Webster says.

To Brian Hoskins, a climate scientist at Imperial College London, the argument of Boos and Kuang rests too heavily on models of vertical air and energy flow in an idealized atmospheric column. It neglects the dynamics of air flow and monsoon structure in the real world, he says: “You probably would get a monsoon without Tibet, but it would look very different. The issue at stake is how the plateau affects monsoon onset, duration, and rainfall distribution.”

Other critics say that the argument highlights fundamental problems of modeling climate in mountain regions like Tibet and the Himalayas—which is notoriously difficult because of their complex topography and the complicated ways it could affect climate. In addition, the models used by Boos

and Wu have a resolution of 200 kilometers, which are too coarse to separate out the effects of the Himalayas and the Tibetan Plateau or to delineate the details of heat and moisture distribution, says Moetasim Ashfaq, a climate scientist at Oak Ridge National Laboratory in Tennessee. The models’ coarseness means that the plateau can’t be fully removed from simulations, Ashfaq says. “It’s difficult to know exactly what you are seeing in such models,” he says. Boos and Wu acknowledge that the coarse resolution is a major constraint.

Ashfaq says that the Weather Research and Forecasting Model, a relatively

heat the Tibetan Plateau, the monsoon starts earlier and you get more rain,” Molnar says. The effects are mostly at the beginning and at the end of the monsoon season. “But we don’t see much of a correlation when we look at the main part, between June and August, when the rain is the heaviest,” he says. According to their study, variations in heating over Tibet might affect a third of the monsoon season and account for only 30% of total rainfall.

Others say that the jury is still out on which model, if any, is correct. Yang Kun, a climate scientist here at the Chinese Academy of Sciences’ Institute of Tibetan Plateau



Weather forecast. Unraveling the monsoon’s cause should sharpen predictions of its start date and duration and how it will respond to climate change.

new regional model that uses massive computing power to simulate processes at 10-kilometer resolution, is superior to global climate models for such simulations. Moreover, the model is nonhydrostatic—meaning that it factors in vertical movement of air and moisture—and is more capable of simulating cloud formation, says Matthew Huber, a climate scientist at Purdue University in West Lafayette, Indiana. When Ashfaq removed the Tibetan Plateau in this model, leaving just the Himalayas, the monsoon’s northern branch “is all gone,” he says. “What the topography does is bring moisture from the equator further inland,” says Ashfaq, who is writing up the results for submission.

Ground truths

Those in the barrier effect camp concede that Himalayan heating plays at least some role in monsoon formation, but maintain that the low-lying plains in northern India are the primary driving force for the downpours.

Molnar and colleague Balaji Rajagopalan, also at the University of Colorado, correlated heating of Tibet and monsoon characteristics in a report in February in the *Journal of Geophysical Research: Atmospheres*. “If you

Research, notes that the plateau is notorious for its lack of climate observations, which cripples the predictive power of global climate models for the region. “Even basic weather stations are very sparse, let alone data on soil moisture, which is the most important parameter for energy exchange between land and atmosphere,” he says. He thinks only high-resolution climate models, ground-truthed with robust long-term data, can hint at the correct answer.

In the past few years, Yang and his colleagues have been gauging soil moisture at more than 100 sites across Tibet. The measurements will be used to calibrate satellite data, allowing scientists to calculate energy flux on the plateau. In addition, China has earmarked \$441 million for stepping up research and establishing state-of-the-art climate observatories and long-term research stations in Tibet and neighboring countries. The data, Yang says, will help elucidate climate processes and validate models. “Until then,” he warns, “any conclusions about the role of the Tibetan Plateau in regional and global climate would be premature.”

—JANE QIU

Jane Qiu is a writer in Beijing.

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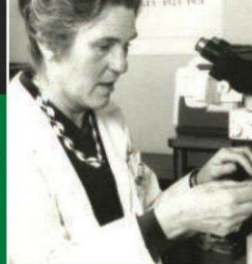
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LETTERS

edited by Jennifer Sills

Risks of Neonicotinoid Pesticides

IN THE NEWS FOCUS STORY "PESTICIDES UNDER FIRE FOR RISKS TO pollinators" (10 May, p. 674), E. Stokstad writes that the use of neonicotinoid insecticides has been partly restricted because of their effects on pollinators. Neonicotinoid insecticides are also a threat to vertebrates due to their high toxicity, environmental persistence, water solubility, and potential for surface- and groundwater contamination (1).

Developed by Shell in the 1980s and by Bayer in the 1990s (2), neonicotinoid insecticides are now the most widely used insecticides in the world because they are less toxic than older insecticides (1). However, when neonicotinoid insecticides bind to the postsynaptic

nicotinic acetylcholine receptor of animals, the resulting excitation can be lethal (3). The American Bird Conservancy reported that a songbird could be killed by a single neonicotinoid-treated corn kernel and that a bird would die after eating a small canola or



Collateral damage. Neonicotinoids harm house sparrows and other songbirds.

wheat grain coated with imidacloprid (a common type of neonicotinoid) (1). The same report suggested that neonicotinoid insecticides have high reproductive toxicity in birds (1). Rats' respiration and behavioral symptoms can be greatly disturbed by neonicotinoid insecticides, raising the possibility that neonicotinoids may also negatively affect human health (3, 4).

In the past, we underestimated the risks of widely used pesticides. As we work to replace older insecticides with less toxic alternatives, we must use caution to prevent a similar mistake.

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Integrity Training: Conflicting Practices

N. H. STENECK RIGHTLY PLEADS FOR GLOBAL research integrity training, although he acknowledges that "[i]mproved and expanded global [responsible conduct of research] training will not necessarily reduce misconduct or improve integrity in research" ("Global research integrity training," Policy Forum, 3 May, p. 552). Research suggests that mentoring and a lab's actual practice of integrity—or the lack thereof—are more important than formal training in research integrity (1).

We analyzed European guidance documents on research integrity and misconduct (2). Most guidance documents propose, without providing much detail, that education in good research practices should be part of research training. However, there is no consensus across Europe about the content, for-

mat, timing, or frequency of such courses, nor is there a common view on who needs training and who qualifies to lead the training. What level of student or researcher should training target? What kind of training could help professors, who heavily influence the culture in which their researchers work (1)? Is there evidence that training adults promotes integrity or prevents dishonest behavior in other areas of life?

Successful guidance for researchers should entail a harmonized strategy to stimulate research integrity. Further research will tell us whether this strategy should focus on training researchers or on the broader goal of creating a culture of integrity in research environments.

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Integrity Training: Misconduct's Source

IN HIS POLICY FORUM "GLOBAL RESEARCH integrity training" (3 May, p. 552), N. H. Steneck writes that research misconduct, to date, has been found to be an intractable problem. He calls for a global research effort using common standards to determine whether improved training is a reasonable way to improve integrity in research.

We should not be surprised to find that Responsible Conduct of Research courses do not influence the behavior of trainees.

Research misconduct—fabrication, falsification, and plagiarism—are the academic equivalents of lying, cheating, and stealing. Ethical standards prohibiting such behavior are established long before students begin graduate training in science.

I reviewed the individual narratives in 146 Office of Research Integrity reports of those found guilty of research misconduct. These accounts suggest that misconduct results from a combination of an individual's character traits, such as perfectionism or sociopathy, and his or her circumstances. For trainees, an intense fear of failure was often the driving

force; for established scientists, it was the lure of academic and/or financial rewards (1).

As a psychiatrist, I know that character traits do not lend themselves readily to remediation, and the anxiety induced by the reality of publish or perish cannot be abolished. However, I believe that we can reduce the prevalence of research misconduct through interventions that decrease the fear of failure in the trainee and increase the fear of detection in the established scientist. Better mentoring and better protection of whistleblowers would accomplish these goals.

Responsible Conduct of Research courses may be effective in teaching good research practices (2), but our efforts to decrease misconduct are more likely to succeed if they are moved from the classroom to the laboratory.

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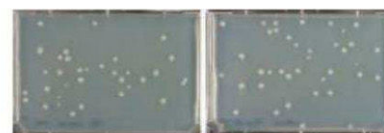
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CORRECTIONS AND CLARIFICATIONS

This Week in Science: "Essential novelty" (7 June, p. 1139). In the final sentence, "centrosome" should have been "centromere." The HTML and PDF versions online have been corrected.

News Focus: "Mysteries of development" by J. Travis (7 June, p. 1156). In the banner caption, the fruit fly embryos pictured show mRNA molecules, not microRNAs. The caption has been corrected in the PDF version online.

Reports: "Killing by bactericidal antibiotics does not depend on reactive oxygen species" by I. Keren *et al.* (8 March, p. 1213). In Figure 2A, a picture of bacterial growth on agar plates was inadvertently duplicated by the authors during revision of the manuscript. The correct version, which was the version in the submitted peer-reviewed manuscript, is shown here. The figure has been corrected in the HTML and PDF versions online. Two panels of fig. S2 were also duplicated, and there was duplication of traces between figs. S2 and S6 in the Supplementary Materials. A correct version of the Supplementary Materials has now been posted, and also includes a correction to the legend for fig. S5. The conclusions of the paper are unaffected.



Letters to the Editor

Letters (~300 words) discuss material published in *Science* in the past 3 months or matters of general interest. Letters are not acknowledged upon receipt. Whether published in full or in part, Letters are subject to editing for clarity and space. Letters submitted, published, or posted elsewhere, in print or online, will be disqualified. To submit a Letter, go to www.submit2science.org.



the**BUZZ**

Impact Factor Concerns

In his 17 May Editorial, "Impact factor distortions" (p. 787), Editor-in-Chief Bruce Alberts endorsed the *San Francisco Declaration on Research Assessment* (DORA), which discourages the use of journal impact factor to judge individual scientists and recommends actions to improve the evaluation system. Many readers wrote in to share their own concerns with the prominence of the impact factor metric, and a few pointed out some benefits. Excerpts of some of those comments are below. Read the full comments at <http://comments.sciencemag.org/content/10.1126/science.1240319>.

A selection of your thoughts:

I agree. True scientific curiosity cannot be guided by forced objectives...

—Suresh Varwandkar

The Editorial is much welcome, especially...in the context of developing nations.... [S]o-called good science, as measured by impact factor, is dominated by science in the developed world....

—Renato de Castro

...The sooner this [evaluation] practice is out-voted in the United States, the more likely that scientists in [other] countries will put some thought behind how to [judge] scientific worth in their own scientific milieu.

—Gaiti Hasan

...[W]ill journals stop emphasizing their impact factors if everyone agrees that we should not consider them for evaluation?...

—Anindita Bhadra

...Since quantitative measures for research and teaching are becoming difficult to assess, the new measure for a professor's success used by tenure committees is the amount of research money brought in by the scientist. The result is that the scientist has become an administrator....

—Parveen Bawa

....[S]cientists are supposed to be role models, mentors, and supervisors for the new generation.... [H]ow can the quality of teaching and supervision be kept high if professors need to mainly focus on producing quantitative metrics?...

—Malgorzata Blicharska

...One further argument against using impact factors to judge individual scientists.... The journal impact factor does not say anything about the impact of any individual paper published in that journal.... The citation count per paper (or derivatives like h-index) is much more informative about a researcher's impact on the field than a metric computed per journal.

—Eelke Spaak

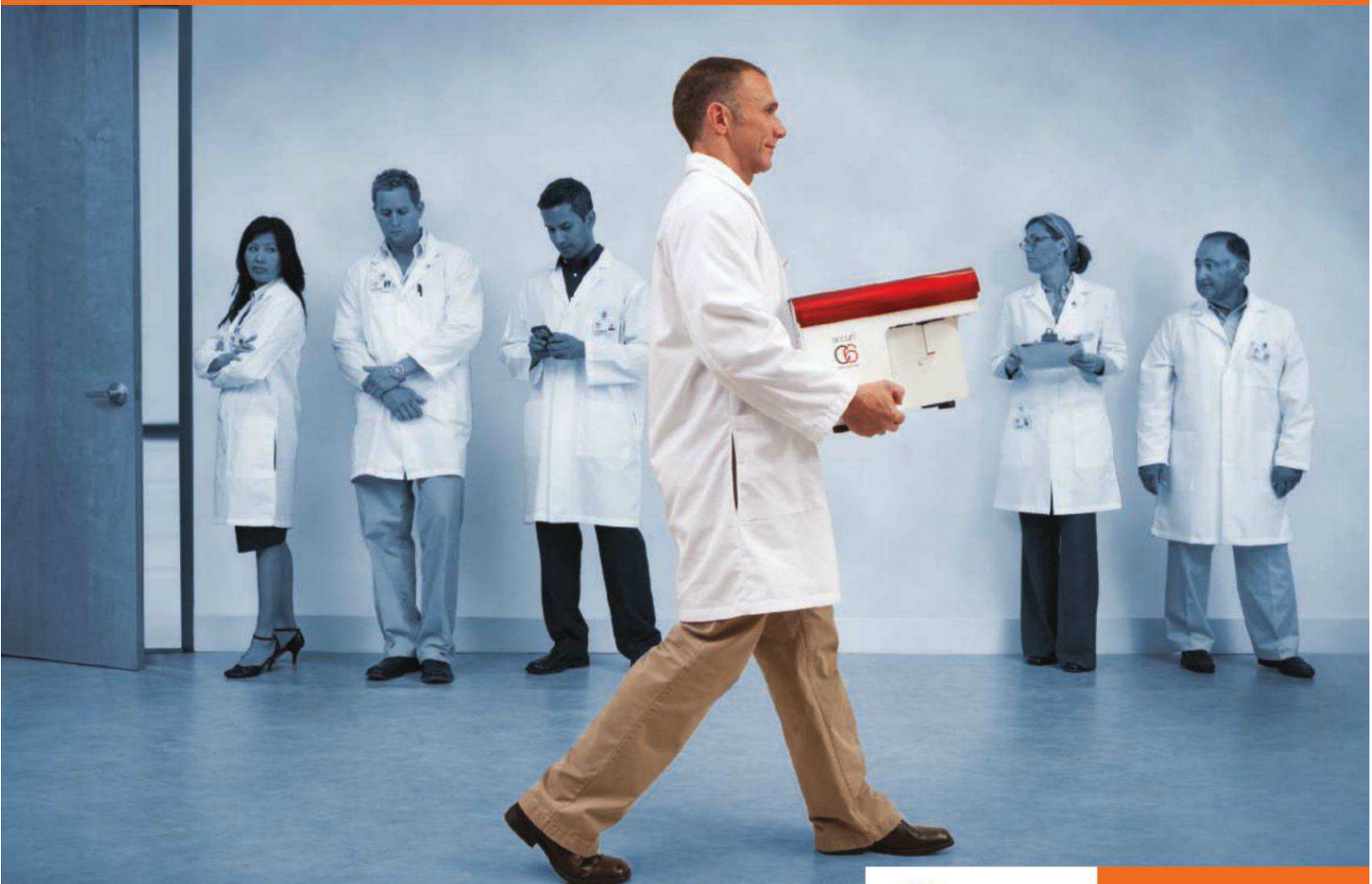
...The impact factor offers a solace to researchers who are troubled by the proliferation of substandard or "predatory" journals, which usually do not have an impact factor.

—Ravi Murugesan

...[T]he impact factor allows committee members to delegate part of their evaluation to the assessment performed by the two or three reviewers who initially accepted the publication.... Committee members should instead delegate their evaluation to all of the true experts in the core field of the assessed publication.... Aggregating in an online database reviews or ratings on the publications that scientists read anyhow can provide important information that can revolutionize the evaluation processes that support funding or hiring decisions....

—Răzvan Valentin Florian

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MENTAL HEALTH

Diagnosing Madness

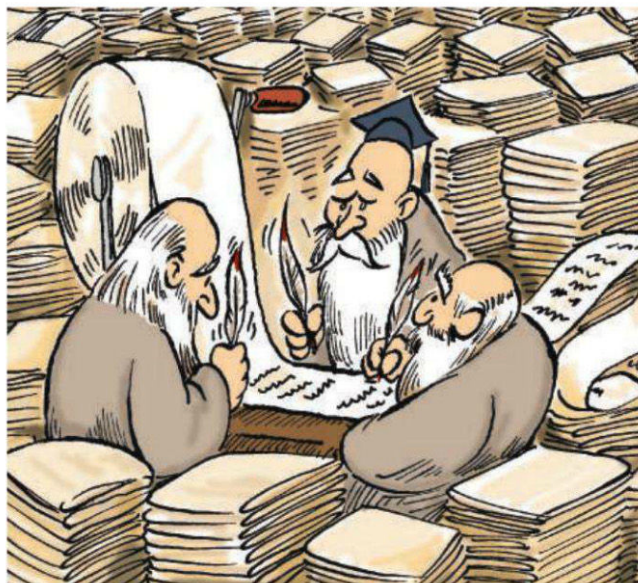
Julio Licinio

According to the influential Brazilian poet Carlos Drummond de Andrade, “The problem is not to concoct. It is to be concocted hour after hour, without ever getting ready our convincing edition” (1). Nothing better describes the lengthy tweaking process that has gone into the making of the fifth edition of *Diagnostic and Statistical Manual of Mental Disorders* (DSM-5) (2), released last month by the American Psychiatric Association (APA).

In *The Book of Woe: The DSM and the Unmaking of Psychiatry*, Gary Greenberg sees this process in more severe terms. A practicing psychotherapist and science writer, Greenberg takes the outsider’s view as someone who neither is a psychiatrist nor treats those suffering from severe, clinical mental illness. As he calls the DSM the “Bible of Psychiatry,” his central premise appears to be based on the starting sentence of the Gospel of John (1:1): “In the beginning was the Word, and the Word was with God, and the Word was God.” Greenberg sees DSM-5 as the “Word” from the APA. In Greenberg’s view, if it can be negated, then the entire profession of psychiatry is fatally flawed. Repeating a frequently voiced complaint, he insists that “without a working nosology [classification of diseases], psychiatry is a failure.”

The book represents Greenberg’s exhaustive attempt to document problems with DSM-5 and the process that led to it as a means of demonstrating that psychiatry “has something rotten at its foundation: its have-it-both-ways, real-until-it-isn’t diagnostic manual.” At the end of the book, he proposes that “[a]n honest psychiatry might also lead the way to a new understanding of illness,” one that recognizes that “[t]he line between sickness and health ... is not biological but social and economic.” Greenberg claims “there is one definition of mental disorder that is not bullshit. Mental disorder, like all disease, is suffering that a society devotes resources to relieving.”

Psychiatry is a branch of medicine. To become a psychiatrist, one must graduate from medical school and then complete years of specialized residency training, a path that



parallels those for all other medical specialties. All psychiatrists are medical doctors, medicine being our profession. Greenberg, however, sees psychiatry not as a branch of medicine but as a self-standing profession that is dishonest because its foundation (laid out in the DSM) is faulty. Early in the book, he claims that “our inner lives are too important to leave in the hands of doctors: because they don’t know as much about us as they claim, because a full account of human nature is beyond their ken.” Later, he writes “of a dream that can’t be fulfilled: to make psychiatry into just another specialized branch of medicine.” And near the end of the book, Greenberg further declares:

You don’t have to be a hater to think that the DSM, no matter how often it is revised or how competently, will never manage to pour the old wine of human suffering into the new skin of scientific medicine. And you don’t have to resort to biblical analogies to show that the Bible of psychiatry is failing to do what it is presumably intended to do, and what would bolster the argument for bringing our mental suffering under the medical gaze: to improve psychiatric treatment.

In that context, Greenberg compares the APA to companies (such as “General Motors, Kodak, and Xerox”) that overplayed their hand when facing major challenges, failing to remember that “confidence is always built on air.” He sees confidence that doctors have the

knowledge to recognize what is and what isn’t a mental disease as crucial to the APA: “Without this confidence, who will buy the book? And without the book, who will believe the psychiatrists? And without belief, how will their treatments work?” It also seems clear that he sees psychiatry as needing to be saved. Nowhere in the book does Greenberg acknowledge the progress achieved by modern psychiatry as evidenced by millions of patients whose mental illnesses are now in remission.

Readers will find that the book mixes together complex topics such as the science of classification; psychiatric nosology; the challenge of dissecting mild emotional, cognitive, and behavioral symptoms that are due to the vicissitudes of life versus those that may constitute mental illness; the social and psychological impact of shifting psychiatric diagnoses; overdiagnosis leading to overtreatment; conflict of interest; human vanity; and academic arrogance.

Moreover, Greenberg peppers the text with reports of multiple interactions between those who created earlier manuals (DSM-III and particularly DSM-IV) and the team that put together the fifth edition. These reports do not follow any type of sequence; they are not organized chronologically or topically. In the absence of a clear plot, the intricate details of myriad personal interactions make the book very unfocused.

Greenberg makes liberal use of the critique of DSM-5 offered by the architects of DSM-IV. But after reading the book I remain uncertain as to whether he agrees with them—and simply recommends that we stick with DSM-IV—or whether he takes a far more radical position and advocates the full eradication of diagnostic efforts by the APA. He portrays the APA as being in dire need of the revenue to be brought in by DSM-5 for its financial bot-

The Book of Woe
The DSM and the
Unmaking of Psychiatry
by Gary Greenberg
Blue Rider Press
(Penguin), New York, 2013.
411 pp. \$28.95, C\$31.
ISBN 9780399158537.

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tom line, but he presents only very inaccurate data to support such a serious claim. In many places—as when he states that “[p]sychiatry is not inevitable [and] not baseball, either”—it appears that he wants profound changes. But he never presents alternative suggestions for the nosology of mental illness. Such classification may be unnecessary in Greenberg’s own suburban psychotherapeutic practice (as described in the book), but it is certainly required for the diagnosis and treatment of clinical psychiatric disorders such as schizophrenia and bipolar disorder.

Diagnostic categories that are based on arbitrary sets of descriptive criteria agreed on by panels of experts but that do not capture underlying biological and psychosocial causes severely hold back psychiatry. Without effective diagnosis, much clinical and treatment research is uninformative. Psychiatric diagnosis must evolve from purely descriptive agreement among experts toward causation. Moreover, it is highly unlikely that there are hundreds of distinct mental disorders, as listed in DSM-5. Psychiatry’s catch-22 is that the current descriptive nosology may actually hinder the elucidation of causation. Nonetheless, DSM-5, with all of its limitations, is simply a tool. The critical issue is how the tool is used.

DSM-5 certainly warrants a deep, insightful critique from an erudite expert who can communicate well both to fellow professionals and to the public at large. Sadly, Greenberg is not that expert and *The Book of Woe* is not that critique. Although Greenberg lost himself in the trees of the DSM-5—going to extraordinary lengths to be part of DSM-5 field trials and developing years-long relationships with key players in the saga of its making—his account reveals two major shortcomings. One is the author’s lack of biological expertise. The neuroscience seems cited as an afterthought by someone who researched the words but cannot articulate them.

The second major failing is equally difficult to overcome. Even if readers pass over the multiple problems of bias, inconsistencies, excessive personal detail, and insufficient expertise, they face a major obstacle to finishing the book. Similar to a singer who chooses a great orchestra and an attractive repertoire but cannot carry a tune, Greenberg chose the key topic of diagnosing madness but is not a gifted writer. An avid reader, I find myself unable to put down anything from the classics to thrillers. I’m particularly drawn to provocative books that challenge my view of the world, and having nothing to read tortures me. Yet after receiv-

ing the bound galleys of *The Book of Woe*, I had to carry the work with me for months just to get through it. Going through more than a few pages at a time seemed painful. The book sorely lacks an insightful and coherent narrative that would captivate readers and make turning pages inevitable.

Those not repelled by the absence of narrative engagement will find *The Book of Woe* strident, gossipy, angry, and inconsistent. We will have to wait for a much-needed scholarly and well-written critique of DSM-5—an account that accurately reflects the concocting of psychiatric diagnoses, which is still ongoing “without ever getting ready our convincing edition.”

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10.1126/science.1236286

CANCER

Tracing a Translocation’s Impact

Aditi Nadkarni

Most of us love an underdog’s success story. Jessica Wapner’s *The Philadelphia Chromosome* includes several such stories. Wapner, a freelance science writer, describes the path from the first description of a chromosomal abnormality in cancer cells to the successful deployment of a gene-targeted medicine against what had previously been a lethal leukemia. Along the way, she pays homage to various scientific underdogs: a graduate student who spent 10-hour days peering through a microscope for the sheer love of observation; a postdoctoral fellow who risked “the judgment and ridicule of [his] peers”

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in negating the findings of a world-famous scientist; a junior researcher who doggedly pursued a goal dismissed as unfeasible; a physician who urged a drug company to set aside commercialism for a cure; and, most important, a patient brought back from the brink of death by a new cancer drug. Wapner aptly compares the investigators to “a hundred painters applying brushes to a canvas at some time or another over twenty-five years, driven only by curiosity and, sometimes, a vague hope that their work might eventually be relevant to human cancer.” Without knowing what the end result would look like, she notes, they ended up creating a “scientific masterpiece.”

A half century ago, a diagnosis of cancer was devastating because it meant death was likely imminent. Most available treatments were ineffective and often put patients through immense suffering before they succumbed to their disease. One such cancer was chronic myeloid leukemia (CML), which rendered patients sallow as it rapidly poisoned their immune systems, flooding them with abnormal white blood cells. The survival rate was a mere 14%, and most patients died within six years. In 1960, Peter Nowell and his graduate student David Hungerford noticed a genetic abnormality in a chromosome of CML patients (1), a discovery that eventually led to the idea that rogue genes could be a cause of cancer.

The genetic abnormality, the Philadelphia chromosome, would become the first genetic defect identified as a direct culprit in cancer initiation.

Wapner’s narrative begins with the discovery of the Philadelphia chromosome and continues through the 30 years that it took to develop a drug that successfully targeted this genetic abnormality, thus turning a once fatal disease into a manageable condition that can be treated with a pill. Today, 12 years after the drug, imatinib mesylate (Gleevec), entered the market, the survival rate of

The Philadelphia Chromosome
A Mutant Gene and the Quest to Cure Cancer at the Genetic Level

by Jessica Wapner
The Experiment, New York, 2013. 327 pp. \$25.95.
ISBN 9781615190676.



Diagnostic abnormality. The reciprocal translocation responsible for chronic myeloid leukemia moves the *abl* gene from chromosome 9 (the larger, which is extended) and fuses it with *bcr* to produce the mutant gene *bcr/abl* on a truncated chromosome 22 (the Philadelphia chromosome).

BROWSINGS

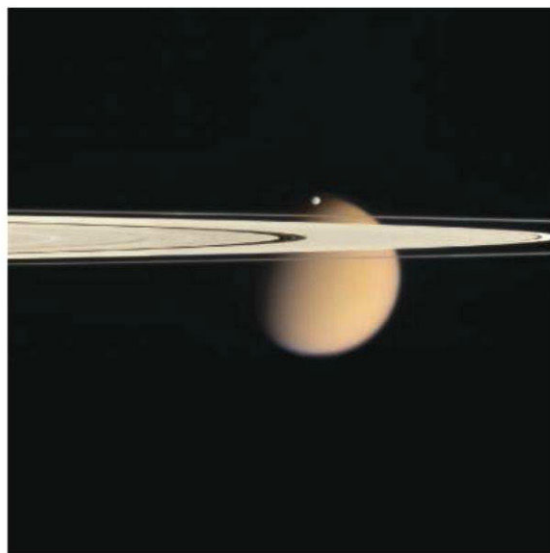
Planetfall: New Solar System Visions. Michael Benson. Abrams, New York, 2012.

210 pp. \$55, C\$62, £35. ISBN 9781419704222.

Using the book's large page size and gatefolds, Benson offers a "subjective compilation" of views of Earth, our Moon, the Sun, Mars, Jupiter, and Saturn (plus two asteroids and a comet) returned by spacecraft between 2000 and 2012. He created most of these striking and revealing photographs by digitally assembling raw image data he drew from the archives of NASA, the European Space Agency, and the Japanese Aerospace Exploration Agency. This mosaic composite shows tiny Epimetheus, giant Titan, and Saturn's A and F rings.

Revolutions in Twentieth-Century Physics. David J. Griffiths. Cambridge University Press, Cambridge, 2013. 184 pp. Paper, \$29.99, £18.99. ISBN 9781107602175. Adobe eBook, \$24. ISBN 9781139848572.

Nonspecialists and nonscientists curious about such topics as spacetime, the EPR paradox, chromodynamics, and the origins of matter will find this concise account a helpful introduction. Despite the title, Griffiths focuses on findings rather than events of 20th-century physics. He reconnoiters relativity, quantum mechanics, elementary particles, and cosmology. Worked examples and scattered problems serve to reinforce readers' comprehension.



CML patients is 95.2%. Told in the manner of a thriller with a dash of history, *The Philadelphia Chromosome* describes the events that went into the "rational design" of that pharmaceutical treatment for CML.

In the early 1970s, geneticist Janet Rowley, using recently developed chromosome banding techniques, observed that the lopped-off head of the Philadelphia chromosome in CML patients had somehow perched onto another chromosome. Going against established views about cancer, Rowley argued that this chromosomal translocation could be the cause of the cancer. Her finding opened the door to the discovery of the cancer-causing oncogene affected by this change in CML patients. It took several years and the concerted efforts of many researchers to uncover the precise mechanism by which the translocated Philadelphia chromosome causes the brakes to fail on a runaway kinase, resulting in uncontrolled cell division—a hallmark of cancer. The discovery of this culprit oncogene, as Wapner describes, ultimately revealed the relation between the Philadelphia chromosome and CML and presented the kinase as a target for therapy. A specific kinase inhibitor could bind to and stop the uncontrolled kinase in its tracks, "[I]like a gloved hand fitting perfectly over a mouth to block the next breath."

Much of the second half of the book focuses on the frantic struggle to get the life-saving drug to patients. Here Brian Druker, a clinical oncologist turned researcher, emerges as a hero through his inspiring commitment to patients and his unyielding determination to save people from the clutches of a fierce disease.

Reading Wapner's account suggests that while the scientists involved in the discovery

and development of Gleevec were underdogs in their field, the drug was somewhat of a stepchild for the company. "There were too few patients with CML to make developing this new drug worthwhile for the company." In short, the drug would not profit the company as much as one that targeted some disease affecting a larger population. This problem of market-driven policy remains a subject of debate. Drug companies are often criticized for shifting their research focus to diseases with a higher incidence to ensure that they have a larger demand for their products.

The author covers a lot of ground in describing the politics of science and dissecting the inner workings of a pharmaceutical company's drug development process. She withholds judgment, instead providing two sides of the story: In one, a physician coaxes a drug company focused on commercial interest to think about patients. In the other, a drug company, bombarded with sudden demand from the market, rushes to complete the toxicity studies required for Food and Drug Administration clearance. Wapner also describes the impact of the CML community's 1999 online petition urging increased production of the drug for clinical trials.

In the last chapter, Wapner surveys the current landscape of cancer research, noting hurdles to continued progress such as difficulties in sequencing tumors and the unintended effects of technology transfer agreements on collaborations. She also mentions the pressing issues of poverty, limited access to medical care, and ever-rising drug prices that patients face. In her concluding comments she notes that although targeted drugs have changed cancer treatment and "medicine as a whole," their failure "to transform

other cancers into tolerable chronic conditions has generated skepticism about the future of the approach."

As a young cancer researcher, I especially appreciate Wapner's mention of the current abysmal funding situation—which does not reward new or risky ideas and is extremely stingy in supporting those early in their careers. She points out that in 2010, less than 4% of the U.S. National Institutes of Health's R01 funding went to scientists under age 36. On the contrary, in the 1960s and 1970s (when many of the discoveries described in the book occurred), being a young scientist with novel ideas was an asset. Some key contributions to the Philadelphia chromosome story, as the author notes, came from young researchers given the freedom to pursue their own ideas. Support for such freedom of thought and creativity is now hard to come by in academic bioscience.

Wapner's use of analogies to describe difficult scientific concepts makes her narrative accessible to lay readers. Still, the jargon in some portions that detail experimental hurdles may overwhelm those lacking firsthand experience in the laboratory. Nonetheless, I appreciate that her artful storytelling does not dilute the complexity of the science or sensationalize the subject. By recounting personal stories of the researchers and patients, Wapner helps readers identify with the story. *The Philadelphia Chromosome* offers powerful testimony on what the cancer research and pharmaceutical fields are capable of when academic scientists, physicians, and drug companies work together.

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10.1126/science.1240366

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PNAS Congratulates 2012 Cozzarelli Prize Recipients

The *Proceedings of the National Academy of Sciences* (PNAS) has selected six outstanding articles for the 2012 Cozzarelli Prize, in recognition of their scientific excellence and originality. Winners were selected from the 3,700 research articles published in PNAS in 2012 and represent exceptional contributions to the six broadly defined classes under which the National Academy of Sciences is organized.

2012 Cozzarelli Prize Recipients

CLASS I: PHYSICAL AND MATHEMATICAL SCIENCES

Water, plants, and early human habitats in eastern Africa

Clayton R. Magill, Gail M. Ashley, and Katherine H. Freeman

(2013) PNAS 110:1175–1180

CLASS II: BIOLOGICAL SCIENCES

Eight pairs of descending visual neurons in the dragonfly give wing motor centers accurate population vector of prey direction

Paloma T. Gonzalez-Bellido, Hanchuan Peng, Jinzhu Yang,

Apostolos P. Georgopoulos, and Robert M. Olberg

(2013) PNAS 110:696–701

CLASS III: ENGINEERING AND APPLIED SCIENCES

Point process modelling of the Afghan War Diary

Andrew Zammit-Mangion, Michael Dewar, Visakan

Kadirkamanathan, and Guido Sanguinetti

(2012) PNAS 109:12414–12419

CLASS IV: BIOMEDICAL SCIENCES

Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling

Xiaojun Lian, Cheston Hsiao, Gisela Wilson, Kexian Zhu, Laurie B.

Hazeltine, Samira M. Azarin, Kunil K. Raval, Jianhua Zhang, Timothy

J. Kamp, and Sean P. Palecek

(2012) PNAS 109:E1848–E1857

CLASS V: BEHAVIORAL AND SOCIAL SCIENCES

Evolution of music by public choice

Robert M. MacCallum, Matthias Mauch, Austin Burt,

and Armand M. Leroi

(2012) PNAS 109:12081–12086

CLASS VI: APPLIED BIOLOGICAL, AGRICULTURAL, AND ENVIRONMENTAL SCIENCES

***Arabidopsis* synchronizes jasmonate-mediated defense with insect circadian behavior**

Danielle Goodspeed, E. Wassim Chehab, Amelia Min-Venditti, Janet

Braam, and Michael F. Covington

(2012) PNAS 109:4674–4677

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Podcast interviews with the authors will be available at www.pnas.org/cozzarelliprize.

Targeting Transparency

David Weil,^{1*} Mary Graham,² Archon Fung²

When rules, taxes, or subsidies prove impractical as policy tools, governments increasingly employ “targeted transparency,” compelling disclosure of information as an alternative means of achieving specific objectives. For example, the U.S. Affordable Care Act of 2010 requires calories be posted on menus to enlist both restaurants and patrons in the effort to reduce obesity. It is crucial to understand when and how such targeted transparency works, as well as when it is inappropriate. Research about its use and effectiveness has begun to take shape, drawing on social and behavioral scientists, economists, and legal scholars. We explore questions central to the performance of targeted transparency policies.

Targeted transparency differs from broader “right-to-know” and “open-government” policies that span from the 1966 Freedom of Information Act to the Obama Administration’s “open-government” initiative encouraging officials to make existing data sets readily available and easy to parse as an end in itself (1, 2). Targeted transparency offers a more focused approach often used to introduce new scientific evidence of public risks into market choices. Government compels companies or agencies to disclose information in standardized formats to reduce specific risks, to ameliorate externalities arising from a failure of consumers or producers to fully consider social costs associated with a product, or to improve provision of public goods and services. Such policies are more light-handed than conventional regulation, relying on the power of information rather than on enforcement of rules and standards or financial inducements.

Transparency has been used as a policy tool at least since the 1906 Pure Food and Drug Act, to improve food safety, and the 1933 and 1934 Security and Exchange Acts, to reduce financial malfeasance. Study of targeted transparency as a distinct form of social policy is relatively new (3). We found that effective targeted transparency policies follow a demanding “action cycle” of information provision, use, and response (4). Con-

sumers must see and comprehend new information and integrate it into choices of products and services; target companies must perceive and act on consumers’ responses in ways that reduce risks, improve services, minimize corruption, or otherwise further a policy goal (4). Third parties may play critical roles, translating complex information into a form more readily used by individuals in market settings or acting through political or other nonmarket channels in response to disclosed information.

Many policies fail to satisfy these conditions. Consumers may not understand or be interested in new information, may not have the capacity to choose when given information, or may not act in accord with policy-makers’ aims. In cases where disclosure relies upon more collective responses, such as pollution emissions, third parties may have agendas not well aligned with the individuals they purport to represent. Target companies may not understand consumers’ changed choices, or, if they do, many not act in accord with policy-makers’ aims. The disclosure mandate itself may be flawed. As in other regulatory regimes, politics plays a role in framing targeted transparency; thus, information completeness, format, or timeliness may be compromised.

Disclosure systems erode over time if not designed to evolve as markets and scientific findings change. Ineffective disclosure requirements can be costly. Forcing companies to collect and disclose information can require substantial resources. Mandated disclosure of incomplete or out-of-date information can mislead consumers and create new risks.

Three Emerging Questions

When should government require disclosure? Some argue that government has only a small role to play in mandating transparency because market pressures create sufficient incentives for businesses to provide accurate information to consumers (5). But multiple consumer priorities—for example, new car prices, fuel economy, and safety—can make revelation of any single dimension skew consumer choices in ways that do not advance public goals (see the chart). Firms may underdisclose because disclosure is costly relative to private benefits. Consum-

Research explores how policy goals might be reached by simply requiring that information be made public.

ers may lack adequate baseline information about quality characteristics and variation to compare products and practices. The case for public intervention arises when such barriers to voluntary disclosure deprive the public of critical information about products or services (5, 6). This information asymmetry pushes markets away from socially optimal outcomes (7).

Even then, government-mandated transparency may not be appropriate or feasible. When no consensus metrics exist, when risks cannot be clearly communicated, or when agencies lack capacity to frame or oversee a targeted transparency system, policy-makers must search for other approaches. Standards- or market-based approaches may be more useful when policy-makers seek specific outcomes like adoption of certain safety practices. Banning substances may be a more appropriate approach when transparency would produce variable responses that perpetuate unacceptable hazards to the public, such as allowing mercury in food.

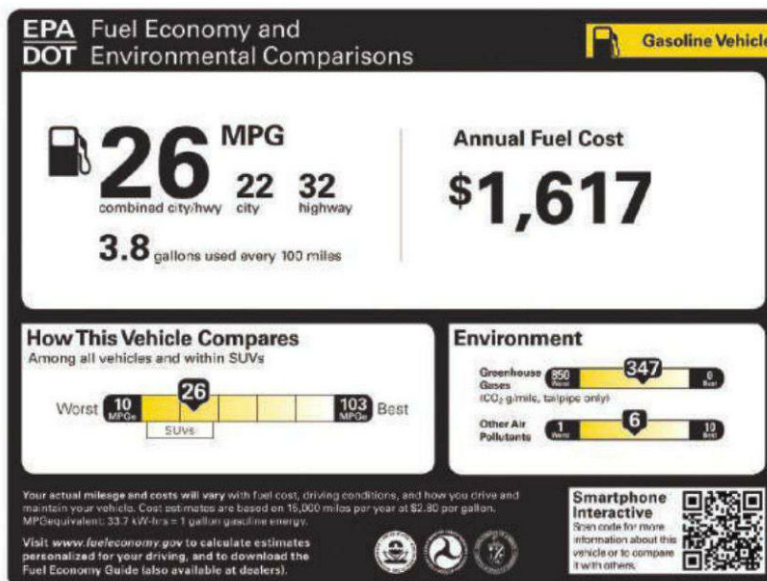
When and how do consumers respond to new information? Research suggests that people use cognitive “short-cuts”—streamlined paths of decision-making that can be useful but, at times, misleading. Individual failures to use or incorporate new information reflect cognitive errors because they lead to suboptimal decisions (8). For example, people may overreact to risks when they feel little control (e.g., flying in an airplane) but underestimate risks when they perceive control (e.g., riding a bicycle). The extent of such problems, and the influence of transparency formats in overcoming them, remains contentious. However, most acknowledge that some choices will be more affected by new information than others, such as decisions that are infrequent and involve unfamiliar alternatives and multiple priorities (e.g., a major medical procedure).

Research suggests that transparency systems can be designed to mitigate such problems. Some policies require user-friendly formats to make complex information comprehensible, e.g., five-star ratings for auto safety or restaurant hygiene grades. Some of these rating systems have proven highly effective (9). In some instances, nonprofit or for-profit third parties may be better equipped and more facile than government in translating complex information into usable formats. Private

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Updated labels for vehicle fuel efficiency and environmental impact. Adopted in 2011 by the U.S. Environmental Protection Agency and Department of Transportation, these illustrate key components of effective transparency: provide information on the policy objective in multiple forms, based on an understanding of how consumers think about fuel efficiency; provide a sense of how a car performs compared with other vehicles; and incorporate scientific information regarding environmental impacts not included in older labels.



organizations rank schools; environmental groups rank polluters; consumer groups rank airline performance.

What factors influence whether target companies respond to disclosure by reducing risks or improving their practices? Recent research has investigated cases in which targeted transparency creates inappropriate incentives for businesses by focusing on one set of quality or risk dimensions versus others. When hospitals are compelled to disclose some, but not all, aspects of patient safety, for example, their actions may increase risks that are not reported or may discourage admission of high-risk patients (10). Companies or agencies may also try to avoid detection, appear as if they have complied or improved their performance, or raise some measures without altering underlying outcomes (e.g., “green washing,” which appears to promote better environmental practice but actually changes very little). As with any high-stakes policies, transparency may lead to attempts to undermine the system politically or to bribe officials (11). Research also suggests the importance of preemptive responses by managers. When required to disclose toxic pollution, some chemical companies took steps to reduce such pollution before the public and press responded (12). Studies of the required disclosure of drinking water contaminants reveal preemptive responses by water-quality agencies (13).

Future Transparency Research and Policy

As research continues on these questions, scholars and policy-makers must also focus on issues that have been neglected.

When to employ transparency versus other policy tools. Some recent policies aimed at discouraging consumption of food and drinks that contribute to obesity have, for example, introduced transparency measures,

whereas others have favored taxes and product bans. But little is known about the relative effectiveness of these actions. Socially beneficial defaults—“nudges”—may be useful when individuals invest little time in understanding risks associated with their choices, but researchers understand little about when those circumstances occur and the marginal efficacy of additional information (14, 15).

How to design policies that improve over time. Our research suggests that some transparency policies become more robust and effective over time, whereas others are reduced to costly and irrelevant disclosure exercises. Policies lose value when dynamics like gaming and regulatory capture undermine their purposes. They gain value when constituencies develop to fortify them or when companies gain competitive advantages from disclosures. Policies must evolve to account for advances in scientific knowledge and economic innovation. Recent transparency provisions designed to stabilize the financial system, for example, will become obsolete unless they are updated to address innovations in securitization, insurance, and derivative financial products.

How to harness information and communications technology. Traditional transparency policies have relied on limited and often outdated information, with data about auto-safety defects, drug side effects, or food contaminants making their way to government offices only slowly. Many incidents escape current reporting channels.

Entrepreneurial efforts have begun to emerge. Cheap sensors enabled citizens to quickly create accurate maps of radiation levels near the Fukushima nuclear accident site in Japan to counter less-nuanced government

models. Web sites where patients share medication experiences provide clues about drug safety and effectiveness as a supplement to clinical trials. Research should examine ways in which technologically enabled networks can complement or replace traditional transparency policies.

Targeted transparency policies seek to mobilize private decisions and market forces to reduce critical risks, to improve public services, and to minimize corruption. Research from a variety of disciplines, as well as experimentation by federal and state agencies,

remains essential to achieve the promise while avoiding the pitfalls of targeted transparency.

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GENETICS

A Story of Swapped Ends

Janet D. Rowley

It was dubbed the “Philadelphia chromosome,” named after the city where the abnormal chromosome was first described in 1960 (1). Peter Nowell, of the University of Pennsylvania, and David Hungerford, at the Fox Chase Cancer Center, had taken a close look at patients with chronic myeloid leukemia (CML) and found that regardless of sex, they had a very small chromosome. It was a turning point in cancer biology—the beginning of a story that would draw new attention to chromosome abnormalities as a cause of cancer, a phenomenon that still influences our understanding of the disease.

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Podcast interview with author Janet Rowley (http://scim.ag/ed_6139).

To appreciate the importance of the discovery of Nowell and Hungerford, it is necessary to understand the state of biomedical science in the 1950s. The prevailing view from studies of experimentally induced cancer was that chromosome abnormalities were the result of genomic instability in cancer cells, not the cause. It was assumed that loss of DNA from the Philadelphia (Ph) chromosome (originally thought to be a deletion in chromosome 21) included genes that regulate cell growth, thereby leading to unrestrained proliferation of leukocytes. The situation was complicated because some patients with CML lacked the Ph chromosome and surprisingly, they had a shorter survival than did those with a Ph chromosome (2). Nonetheless, the presence of the Ph chromosome became an important diagnostic tool in hematology, and it appeared to be the exception to the established view that chromosome changes were variable and irrelevant in cancer.

The situation changed dramatically in the 1970s when several new staining techniques revealed chromosomes with unique banding patterns (transverse stripes) that allowed them to be distinguished individually and precisely (3, 4). Having learned a banding technique at Oxford University, I returned to the University of Chicago to apply the method to chromosome samples from leukemia patients.

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Distinguishing chromosomes. New staining techniques developed in the 1970s allowed the visualization of characteristic banding patterns on chromosomes. Janet Rowley (shown) used images of banded chromosomes (white on black background) to show that the Philadelphia chromosome in CML was a translocation between chromosomes 9 and 22.

Among these patients were two with acute myeloid leukemia (AML), where banding revealed that a piece of chromosome 8 had broken off and joined chromosome 21. This was the first recurring chromosomal translocation [t(8;21)] to be identified (5). Were chromosomal changes consistent in other leukemias? It was already known that CML patients in terminal blast crisis showed a gain in middle-size chromosomes; these, I discovered, all turned out to be chromosome 8. What was even more startling was that chromosome 9 had an extra piece of material whose staining resembled that of the missing piece of the Ph chromosome (by then, known to be chromosome 22). This suggested that the Ph chromosome could be the result of a translocation involving the swapped ends of chromosome 9 and chromosome 22. Leukemia cells from the same patients in the chronic phase of CML showed the same (9;22) translocation, whereas nonleukemia cells from their peripheral blood had a normal karyotype. It seemed quite likely that the Ph chromosome was an acquired translocation, a finding I reported 40 years ago (6).

Forty years ago, a chromosomal translocation was discovered to cause leukemia and revealed cancer as a genetic disease.

There the matter stood for a decade. In the meantime, Herbert Abelson had isolated a virus that induced B cell leukemia. The “Abelson” virus could transform normal murine lymphocytes and fibroblasts, and the causative viral factor was a protein with tyrosine kinase activity (v-Abl). The human counterpart of the Abelson viral gene, *ABL*, was mapped to chromosome 9 (7). Moreover, the only additional DNA found in the Ph chromosome was from chromosome 9 (8). The laborious task of cloning the chromosomal breakpoint in CML revealed that the *ABL* gene on chromosome 9 was translocated into part of a gene called the breakpoint cluster region (*BCR*) in chromosome 22, creating a *BCR-ABL* gene fusion (9–11).

The only previously cloned translocation breakpoints, namely the t(8;14) in Burkitt lymphoma (B cells), had involved an oncogene called *MYC* (the human counterpart of the viral oncogene *v-myc*), and the immunoglobulin gene on chromosome 14 (12, 13). The discovery that oncogenes were involved in translocation breakpoints proved to be a remarkable validation of virology and of cytogenetics, fields that were struggling to show their relevance to human cancer. That CML involved the human oncogene *ABL* was welcome corroboration. Additional translocations were found to involve oncogenes as well, a few of which encoded tyrosine kinases like *ABL*; others involved genes that activate transcription factors that function in cell growth, differentiation, and even cell death. It was fortuitous that at the same time, drug companies were developing tyrosine kinase inhibitors. From this focus emerged imatinib (marketed as Gleevec), the compound eventually approved in 2001 to treat CML (and later, for other cancers). Like all tyrosine kinase inhibitors, imatinib prevents the protein (BCR-ABL, in the case of CML) from phosphorylating proteins that promote cancer development (14). This pharmaceutical breakthrough came almost 50 years after the discovery of the Ph chromosome. Imatinib changed CML from a disease with a 3- to 5-year average life span to one where patients have an almost normal life expectancy, especially with the advent of new second- and third-generation tyrosine kinase inhibitors. These later drugs, especially ponatinib, have been designed to be

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effective despite mutations in the activation domain of the ABL protein.

Whereas translocations were first identified in leukemias, lymphomas, and sarcomas, they are now cropping up in many common epithelial tumors, prostate cancer, and lung cancer, among others. Next-generation sequencing of leukemias and solid tumors has revealed a host of translocations (often small deletions or inversions) (15), some of which involve genes that are targets of drugs already approved for therapy of other conditions. It took only a few years from the discovery of the *EML4-ALK* translocation in lung cancer to the development of the tyrosine kinase inhibitor crizotinib (16), indicating that the discovery of new translocations may be more rapidly translatable to drug discovery. Although data on the occurrence and types of new translocations, based on karyotype analysis, are more frequently reported for hematologic cancers (75%) than for solid cancers (mainly epithelial) (25%), the proportion of malignancies that have recurring chromosomal transloca-

tions is the same in both. Moreover, the genes involved have the same function in both cases (17). Thus, translocations are remarkably similar in function, though not necessarily in their frequency in individual cancers.

It is likely that next-generation sequencing will reveal a much higher incidence of gene fusions in solid tumors. But this method is a two-edged sword. It has identified numerous chromosomal translocations and deletions, but which of these lead to altered gene function and which are inconsequential? It will be difficult to distinguish them in the future without characterizing RNA from the tumors.

A goal of personalized medicine is to identify virtually all of the targetable genetic and epigenetic abnormalities in a patient's tumor through next-generation sequencing and other technologies. To evolve targeted treatments for cancer, we also need a more sophisticated understanding of tumor-specific antigens and chromatin modifications, for example. There likely will be many surprises along the way, and paradigms will be discarded. Neverthe-

less, the goal will always be the same—to treat disease and benefit the patient.

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PHYSICS

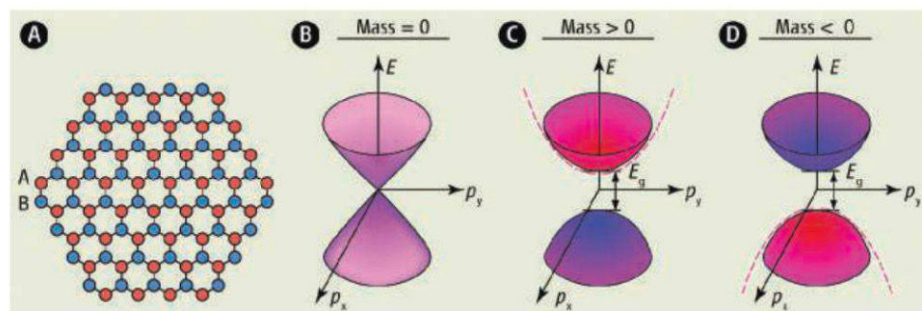
Critical Mass in Graphene

Michael S. Fuhrer

One of the most striking properties of graphene, a single-atom-thick layer of carbon, is that the electrons behave as if they have no mass. They move at a constant velocity, regardless of their energy, much like photons, the more familiar massless particles of light. Special relativity tells us that a minimum energy $E = 2m_0c^2$ is required to create a particle and antiparticle of rest mass m_0 (c is the speed of light; the 2 occurs because two particles are created). Because photons have no rest mass, a pair of photons can be created with energies all the way down to zero energy. In a solid, the band gap energy $E_g = 2m_0v^2$ is the energy required to create an electron and hole (particle and antiparticle), where m_0 is the effective mass and v is the Fermi velocity (typically less than the speed of light by a factor of several hundred). Thus, mass and band gap are intimately related; no mass equates to no band gap, and until now that was the end of the story in graphene. On page 1427 of this issue, Hunt *et al.* (1) show that electrons in graphene can gain a mass under the right circumstances.

The massless property of graphene's electrons is due to the symmetry of the lattice: The simplest repeat unit, the unit cell, has two identical carbon atoms (see the figure, panel A). There are thus two zero-energy states: one in which the electron resides on atom A, the other in which the electron resides on atom B. Both the elec-

Contact with a layer of boron nitride provides a route to control the electronic properties of graphene.



Mass and band gap. (A) Graphene has two atoms in its unit cell, labeled A and B. (B) If A and B are identical (have the same energy), then graphene electrons have zero mass and a gapless dispersion relation [energy E versus momentum (p_x, p_y)]. All electronic states exist equally on both atoms (denoted by magenta in dispersion relation). (C) When the energy of atom A is raised relative to atom B, electron states primarily on atom A (red in dispersion relation) have higher energy than electron states primarily on atom B (blue in dispersion relation) and a band gap E_g is opened. If we examine electron states that reside primarily on atom A (red in dispersion relation), we find a positive curvature of the energy versus momentum relation (red dashed curve) and thus positive mass for these states. (D) When the energy of atom A is lowered relative to atom B, states on atom A have an energy versus momentum relation with negative curvature (red dashed curve) and thus negative mass.

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in the unit cell. Here, the electrons do have a mass and a large (>5 eV) band gap (2), but too large to be technologically useful.

Hunt *et al.* show that hBN in contact with graphene can slightly alter the potential felt at atom A versus atom B, and this effect is large enough that the graphene electrons develop a mass, and hence a band gap (3). In this case the band gap is much smaller, about 30 meV, but large enough to alter graphene's properties in technologically interesting ways.

One curious aspect of the sort of mass and band gap that can be created for massless particles such as electrons in graphene is that the mass can be positive or negative. Consider then an arrangement of graphene on hBN that slightly raises the energy of an electron on atom A relative to atom B such that it has a positive mass (see the figure, panel C). Then likewise an arrangement that raises the energy of atom B should have a negative electron mass (see the figure, panel D). These two versions of graphene-with-a-mass behave alike and would be indistinguishable in an optical spectroscopy experiment. But an interesting thing happens when they come into contact: An electron traveling from a positive-mass region to a negative-mass region must go through an intermediate region where its mass once again

becomes zero (4). This intermediate massless region is gapless and therefore metallic. The presence of such metallic modes at the boundaries between semiconducting regions of opposite-sign mass is a hallmark of a topological phase. "Massive" graphene displays much the same physics as topological insulators (5).

Even if the graphene layer is perfectly aligned in angle to the underlying hBN lattice, a small difference in the atomic spacing in the two materials means that they go in and out of registry over ~ 14 nm. Hunt *et al.* use this property in their massive graphene on hBN to examine the Hofstadter butterfly (6), as have two other groups—one in bilayer graphene on hBN (7), another in graphene encapsulated above and below by hBN (8). The effect results from the interplay of the length scale determined by the repeating overlap of graphene and hBN atoms with another length: the size of electron orbits in a magnetic field. The conductivity of such a sample as a function of these two parameters exhibits a fractal pattern whose butterfly shape gave its name to the phenomenon.

The observation of a controllable mass and band gap in graphene leads to a new way to engineer its properties. Confining graphene electrons is notoriously difficult; for

example, theory tells us that massless electrons will leak out through even the highest barrier. However, if the mass in graphene can be controlled, electrons can be confined to massless regions in graphene by surrounding them with massive regions. This would allow the patterning of quantum dots, wires, and other mesoscopic structures. Intriguingly, generating adjacent areas of opposite-sign mass in graphene will create one-dimensional conductors along the boundary. These wires would be protected against backscattering and could carry currents without dissipation. The ability to control both the magnitude and sign of the mass of charge carriers is fundamentally new and is likely to spawn unanticipated new phenomena.

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ENGINEERING

Controlling the Polarity of Silicon Nanowire Transistors

Thomas Ernst

Each generation of integrated circuit (IC) technology has led to new applications. The most recent advances have enabled noninvasive surgery, three-dimensional (3D) games and movies, and intelligent cars, to name a few. A single chip can contain more than 1 billion elementary devices, and this gain in complexity has been achieved by fabricating nanometer-scale transistors used as switches or memories. Recent experimental work by De Marchi *et al.* (1) describes changes to the structure of one of the most basic bricks of ICs by controlling the type of conduction occurring in vertically stacked silicon (Si) nanowire transistors (see the figure, panels A and B), thus making a programmable transistor.

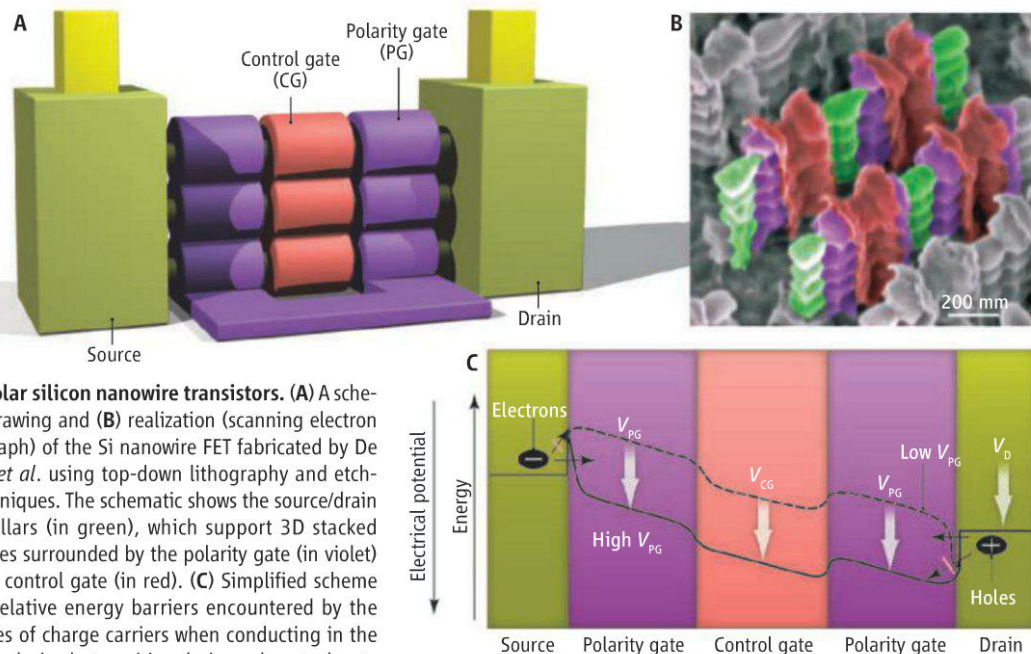
Complementary metal oxide semiconductor (CMOS) field-effect transistors (FETs) are the basic bricks of contemporary electronics. Developed industrially in the 1970s, they enabled the information technology boom because of their scalability—the ability to meet higher performance targets as their size decreased—and their extremely low power consumption. Several physical limits were faced during this half-century history, but each was met by making changes in how to make each component represented by CMOS. The transistor gate electrode was changed from metal to silicon and more recently back to metal. The gate oxide that provides the electrical insulation was changed from silica, which faced quantum-tunneling limits as a barrier, to material with high dielectric constant κ (such as hafnium-based oxide or silicate). The

semiconductor channel itself was changed to increase the current by introducing germanium into silicon, and many alternative materials have been investigated, including ultrathin silicon films, "III-V" semiconductors such as indium gallium arsenide, and carbon electronics, such as nanotubes and graphene.

De Marchi *et al.*, as well as other research groups (2–4), are revisiting how to make "complementary" logic gates. This name comes from the use of two types of transistors, n- and p-type, used in almost every elementary logic gate. The opposite polarity of these transistors is usually determined by the type of impurities (also called dopant atoms) that replace one Si atom out of a thousand or so in the crystalline lattice. Dopants are implanted in the crystal lattice at high energy, and the semiconduc-

tor channel itself was changed to increase the current by introducing germanium into silicon, and many alternative materials have been investigated, including ultrathin silicon films, "III-V" semiconductors such as indium gallium arsenide, and carbon electronics, such as nanotubes and graphene.

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Ambipolar silicon nanowire transistors. (A) A schematic drawing and (B) realization (scanning electron micrograph) of the Si nanowire FET fabricated by De Marchi *et al.* using top-down lithography and etching techniques. The schematic shows the source/drain (S/D) pillars (in green), which support 3D stacked nanowires surrounded by the polarity gate (in violet) and the control gate (in red). (C) Simplified scheme of the relative energy barriers encountered by the two types of charge carriers when conducting in the device polarized at positive drain and control gate (CG) voltages (for simplification, the semiconductor energy band gap between electron and hole energy is not represented). The type of carriers when selected by the polarity gate potential (V_{PG}) can tunnel at the barrier near the metallic source (or drain) and the semiconductor. Adapted from (1).

tor is briefly annealed at high temperature ($\sim 1000^\circ\text{C}$). The dopants provide the type of charge carriers needed for the conduction: electrons (n-type) or holes (p-type), virtual positive charges that represents the displacement of a lack of electrons.

Because n- and p-type devices use opposite charge carriers, they conduct (are in the ON state) for opposite voltages applied on the control electrode (gate) V_{CG} (see the figure, panel C). This association of two complementary devices is required for basic logical functions (performed by logic gates). The simplest example is the NOT function (an inverter) that converts a positive voltage to zero, and vice versa. Digital computing combines these simple operations into many more complex logical operations.

Not all devices are limited to a single conduction mode. Ambipolar MOS transistors conduct with both electrons and holes by replacing the n- and p-type doped regions used as source (S) and drain (D) with a metal that can provide both types of conduction. A metal in contact with a semiconductor creates a Schottky junction, whose conduction depends on the energy barrier naturally created between both sides of the metal-semiconductor contact (see the figure, panel C). A low barrier height is needed at the source side to induce large quantum tunneling currents in the ON state through the barrier.

Meanwhile a relatively high complementary barrier is needed to suppress leakage current from the “reverse” tunneling by carriers of opposite signs at the drain in the OFF state.

De Marchi *et al.* tuned the barrier height in their devices by introducing a polarity gate near a nickel silicide (NiSi)–Si Schottky junction. The barrier height, which is low for the selected carriers in the ON state, is high for the other type of carrier and prevents any parasitic current leakage in the OFF state. Thus, the fabricated device has a polarity gate, which selects the type of carrier (electrons or holes) in the transistor, in addition to the standard control gate that determines conduction.

Previously, two metals have been used to form different (but static) energy barrier heights to block one type of carrier and let the other type through, for example, with platinum silicide for p-type MOSFETs (PMOS) and erbium silicide for n-type MOSFETs (NMOS) (5). Several advantages arise when the Schottky barrier is tuned instead electrostatically (6). First, the new device is “intelligent” as a reconfigurable (n or p) switch but also embodies a logic gate (exclusive OR). This opens new ways to design circuits (7). Moreover, this approach, with only one type of metal, can be useful for several emerging semiconductor materials when the contact technology remains a problem, as already demonstrated for carbon electronics (2–4). In other respects, the elimination of dopants drastically reduces the maximum processing temperature needed, which paves the way toward stacking independent transistor layers, or 3D integration (8).

Nevertheless, many questions should still be answered to make this structure with three gates perform well. If some basic figures of merit for transistors are considered, the additional polarity gates will introduce parasitic capacitances, serial resistances, and area consumption. Thus, logical functions need to be designed completely differently to maintain the targeted performances, such as low consumption and high speed (7). This path has been opened for nanowires by the experimental demonstration by De Marchi *et al.* of new basic logical functions, but

evidence at the circuit level remain necessary.

More generally, multiple-electrode nanowire transistors can be a way to make reconfigurable, highly dense logic gates in the future. Previously, 3D “staked” nanowires have been fabricated with two independent control gates (9). These devices can be used for power management and innovative logical function design. With polarity control gates, new ways of designing arrays of wires in 3D by low-temperature processes could be imagined. For instance, 3D arrays of nanowire transistors are now considered by industry for 3D memories (10). They may be used tomorrow for 3D ICs, and eventually with emerging semiconductor materials.

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GENETICS

Herit-Ability

Jonathan Flint and Marcus Munafò

Studies of the genetic basis of human behavior have a history of generating controversy. For example, when studies of identical and nonidentical twins were first used to estimate the proportion of variation in income and years of schooling that can be attributed to genetic variation (in other words, the heritability of going to university and becoming rich) (1), one response was that the estimates were “pointless” and that determining the heritability of socioeconomic achievement measures should be abandoned (2). Yet, on page 1467 of this issue, Rietveld *et al.* (3) claim progress toward identifying genes underlying variation in educational attainment. How should these findings be interpreted, given that similar claims in the past have often not borne out (4, 5)?

Behavior, like other complex traits (such as height and weight), may arise in part from the combined action of hundreds, if not thousands, of individual tiny genetic effects across the genome (6). Efforts to locate the contributing variants have proceeded through (unsuccessful) family studies involving the search for the coinheritance of trait and genetic markers, and through candidate gene studies (also generally unsuccessful) focused on genes thought to be involved on the basis of known or presumed neurobiology. Genome-wide association studies (GWAS) test whether a trait is associated with variation at any one or more of hundreds of thousands, or even millions, of markers. There is a consensus that for non-behavioral traits, GWAS works well, with the caveats that it only finds a small fraction of the loci involved and does not capture all forms of genetic variation (7, 8). However, with notable exceptions (9), GWAS of behavior has not had the success achieved for GWAS of weight, height, and diseases.

Perhaps this is because behavior is not genetically regulated like other traits. It could be that a large proportion of the predisposition to autism and schizophrenia is due to extremely rare variants, some arising *de novo*, which would be undetectable with GWAS methods (because these use only known, relatively common types of



Achievement genes? Analyses of a genome-wide association study reveal genetic variants that are linked to educational achievement, but each with a very small effect.

genetic variation) (10). Alternatively, the genetic effects on behavior might be even smaller and more numerous than suspected and therefore evade detection (11).

The results of Rietveld *et al.* provide some answers. The contribution of each genetic locus to variation in educational attainment (defined in the study as years of schooling and the completion of college) is particularly small. The effect attributable to a locus is much smaller than for physical traits, and only just detectable with the enormous sample employed (about 126,000 individuals). The largest estimated effect (0.02%) is an order of magnitude smaller than those reported for height (0.4%) (12) and weight (0.3%) (13). However, that finding alone cannot explain the overall contribution of small effect loci. For this purpose, another observation is important.

Suppose that there are only 10 loci contributing to the genetic variation, and that they are the top 10 signals in the GWAS, each contributing 0.02%. If all 10 signals are combined to create a new predictor, one can ask how much of the variation in a new cohort the combined 10 variants explain. This is known as a polygenic score method (14). If the correct 10 variants are selected (and some other assumptions are made about similar allele frequencies), the polygenic score in the new population would be expected to account for about 0.2% of the variance (10×0.02). Now suppose that there are hundreds, perhaps thousands, more variants that contribute. Adding these additional variants to the 10 will increase the ability to explain variation in the trait,

A genome-wide association study reveals possible variants that influence the complex behavior of educational attainment.

whereas if only the 10 variants are contributing, then nothing will be gained, however, many additional variants are combined. As shown by Rietveld *et al.*, the more variants are added, the better the prediction. In short, educational attainment looks to be a very polygenic trait.

But why are the effects so small? Is this just because of the poor quality of the measurement in this, and other behavioral studies (15)? To some extent, Rietveld *et al.* concede this point. They argue that the GWAS effort might progress by capitalizing on the

wide availability of very crude phenotypic measures, such as years of education, to identify genes robustly associated with an outcome of broad social relevance. These genes can then be further analyzed with the knowledge that they are associated with something, even if it isn't known exactly what or how.

The argument that whole-genome methods should inform candidate gene approaches (16) has demonstrated utility. Indeed, heaviness of smoking has been robustly associated with variation on the long arm of chromosome 15, using measures of number of cigarettes smoked that are widely available across a large number of cohorts (9). This phenotype can be very precisely measured, but is nevertheless a very imprecise measure of actual tobacco exposure, largely because of interindividual variability in how those cigarettes are smoked (17). Because nicotine consumption is most likely under the strongest genetic control (18), more direct measures of nicotine (and therefore tobacco) exposure provide a much more precise assessment of the strength of genetic association (15), while at the same time elucidating the likely mechanistic pathway.

The data from years of education make it possible for Rietveld *et al.* to test the importance of trait measurement, because educational attainment was measured in different ways, sometimes with a brief questionnaire, sometimes in more detail. There was no evidence that genetic effects are weaker in cohorts with coarser measures, which the authors argue is consistent with the view

that the “better” measures won’t improve power to find genetic effects. However, cigarette consumption can be measured very reliably (i.e., precisely and accurately), but it still is not necessarily a reliable measure of actual tobacco exposure. Years of education can certainly be measured precisely, but it is still a noisy phenotype given the wide range of attainment and experiences that may result from the same number of years of education. Ultimately, in the case of the study by Rietveld *et al.*, the question remains: What, exactly, is being measured? It seems that a genetic association has been observed for “something,” but exactly what will require considerably more work. The

nebulous nature of the phenotype makes this task considerably more difficult than in the case of heaviness of smoking.

A reasonable assumption is that educational attainment, and the years spent in schooling, partly reflects intellectual ability; those with a higher intelligence quotient (IQ) generally do better at school. So is this, by the backdoor, the first successful study of the genetics of IQ? That will certainly reignite some old disputes.

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CHEMISTRY

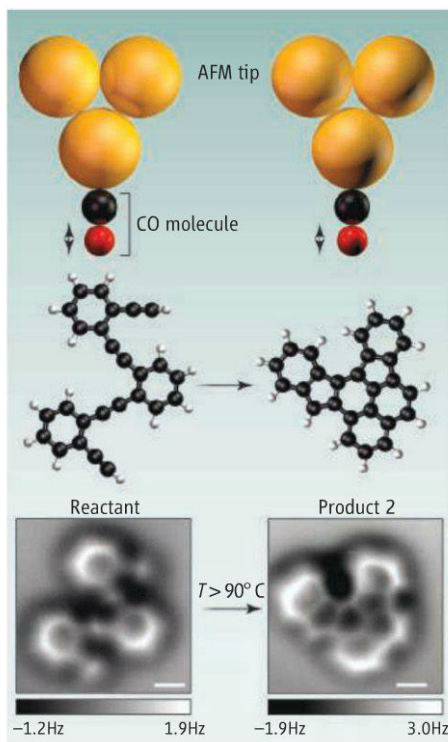
Seeing the Reaction

Franz J. Giessibl

What happens as a molecule goes through a chemical reaction? Model studies have provided important insights into these processes, but it remains extremely difficult to follow all the atomic rearrangements of a chemical reaction experimentally. In many cases, a reaction cannot be observed directly in real space, for example, because the reactants are in the gas state, zooming around at the speed of sound. On page 1434 of this issue, de Oteyza *et al.* (1) report atomically resolved imaging of a complex molecule as it undergoes a chemical reaction on a metal surface.

The authors investigated the molecule phenylene-1,2-ethynylene ($C_{26}H_{14}$) adsorbed on a silver surface. Upon heating above 90°C, the molecule split into several different chemical products (see the figure). The authors imaged several different products with scanning tunneling microscopy and identified them with the help of non-contact atomic force microscopy (2). They calculated the reaction pathway by density functional theory.

Three challenges had to be met to successfully perform this experiment. First, the authors had to find and synthesize a molecule that undergoes a reaction within the experimentally accessible temperature range, with both reactants and products remaining attached to the metal substrate



surface within a viewing field accessible by the microscope.

Second, they had to overcome the “fat and sticky finger problem” (3), which arises because the metal atoms of the probe are large relative to the hydrogen and carbon atoms in organic molecules and exert relatively large attractive forces (4). Meyer and co-workers have found a fine solution to this problem by attaching a carbon monoxide

A molecule is imaged at atomic resolution as it undergoes a chemical reaction.

Before and after. Schematic view of the reactant phenylene-1,2-ethynylene molecule (left) and product 2, one of three different products imaged by de Oteyza *et al.* with atomic force microscopy (AFM). Previous AFM studies focused on imaging individual molecules. Several challenges had to be overcome to extend the method to imaging both the reactant and the reaction products.

molecule to the end of the metal tip, which enabled unprecedented spatial resolution of pentacene, an organic molecule (5). This is the imaging method used by de Oteyza *et al.*

Third, the force sensor that holds the metal tip had to be sufficiently sensitive to probe the tiny forces between the carbon monoxide molecule and the organic molecule to be probed. To obtain their results, de Oteyza *et al.* had to operate their atomic force microscope in the small-amplitude mode and perform highly precise frequency measurements (6). Further challenges were associated with the complex sample and tip preparation and with heating the sample to initiate the reaction and then cooling it to image acquisition temperature. Overall, the work is a masterful experimental achievement.

The interpretation of image data is comparatively simple for flat hydrocarbons [such as pentacene (5) and phenylene-1,2-ethynylene studied by de Oteyza *et al.*], where the submolecular contrast is due to Pauli repulsion between the oxygen atom that terminates the tip and the carbon and hydrogen atoms that constitute the molecule (7). The flat orientation of the molecule results in a

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constant van der Waals background force between the tip and the metal substrate, also simplifying data interpretation. Recently, Gross *et al.* were even able to determine the bond orders within cyclic hydrocarbons and fullerenes with force microscopy (8).

When elements such as nitrogen and oxygen are present, as in the case of cephalandole A (9), image interpretation becomes more complicated, because whereas the carbon monoxide tip is repelled by carbon and hydrogen, it is attracted to nitrogen and oxygen. Atomic identification of single chemical species by force spectroscopy, pioneered by Sugimoto *et al.* (10) for semiconductor surfaces, might become feasible for the chemical analysis of individual atoms in single molecules. This would allow the study of reactions of more complex individual molecules.

The study of molecules that are not flat is also very challenging, because force microscopy senses short- and long-range forces (6), and long-range forces change dramatically with distance. Recently, Repp and co-

workers successfully imaged dibenzo[a,h]thianthrene, a butterfly-shaped molecule with the body flat on the surface and the two wings reaching up from the surface (11). The authors brought the oscillating tip very carefully to the molecule and used a combined method of tunneling current feedback while recording the frequency shift for determining the adsorption site. To extend the method to even more challenging three-dimensional molecules such as DNA, not only the tip-terminating carbon monoxide molecule at the front end would be important but the overall sharpness and crystallographic orientation of the tip would need to be known (12).

The observation of chemical reactions by force microscopy at submolecular resolution reported by de Oteyza *et al.* is of great value in cases where a reaction results in an ensemble of products, as in the decay of phenylene-1,2-ethynylene. It also appears possible to induce the formation of chemical bonds by forces exerted by the tip of the atomic force microscope, similar to reactions induced by tunneling currents (13) or

the flipping of atom dimers on a semiconductor surface (14). Twenty-seven years after Binnig, Quate, and Gerber introduced the atomic force microscope (15), it remains exciting to watch what this technological marvel holds in store for the future.

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10.1126/science.1239961

SOCIOLOGY

A Theory of City Size

Michael Batty

As cities get bigger, they generate economies and diseconomies of scale, referred to by Marshall more than a century ago as the effects of agglomeration (1). Simple theories assume that cities exist due to a trade-off between these positive and negative forces of agglomeration and that the benefits continue to outweigh the costs of cities as they grow ever larger (2). But precisely what happens as cities grow? On page 1438 of this issue, Bettencourt (3) shows that the ways in which people interact with one another in cities lead to power laws—or allometric laws—that relate population, area, and attributes of the population to scale.

Bettencourt *et al.* have previously demonstrated the qualitative changes associated with the scale of cities (4). The larger a city, the greater the benefits with respect to attributes such as income earned, but also the greater the costs with respect to social interactions such as crime (4). There are also gains in efficiency of infrastructure provision, because as

cities get larger, they use less space per capita for utilities, transport routes, and residential living. These allometric laws are demonstrable for socioeconomic attributes such as income, the production of patents, financial services, and crime, all of which scale superlinearly with respect to population.

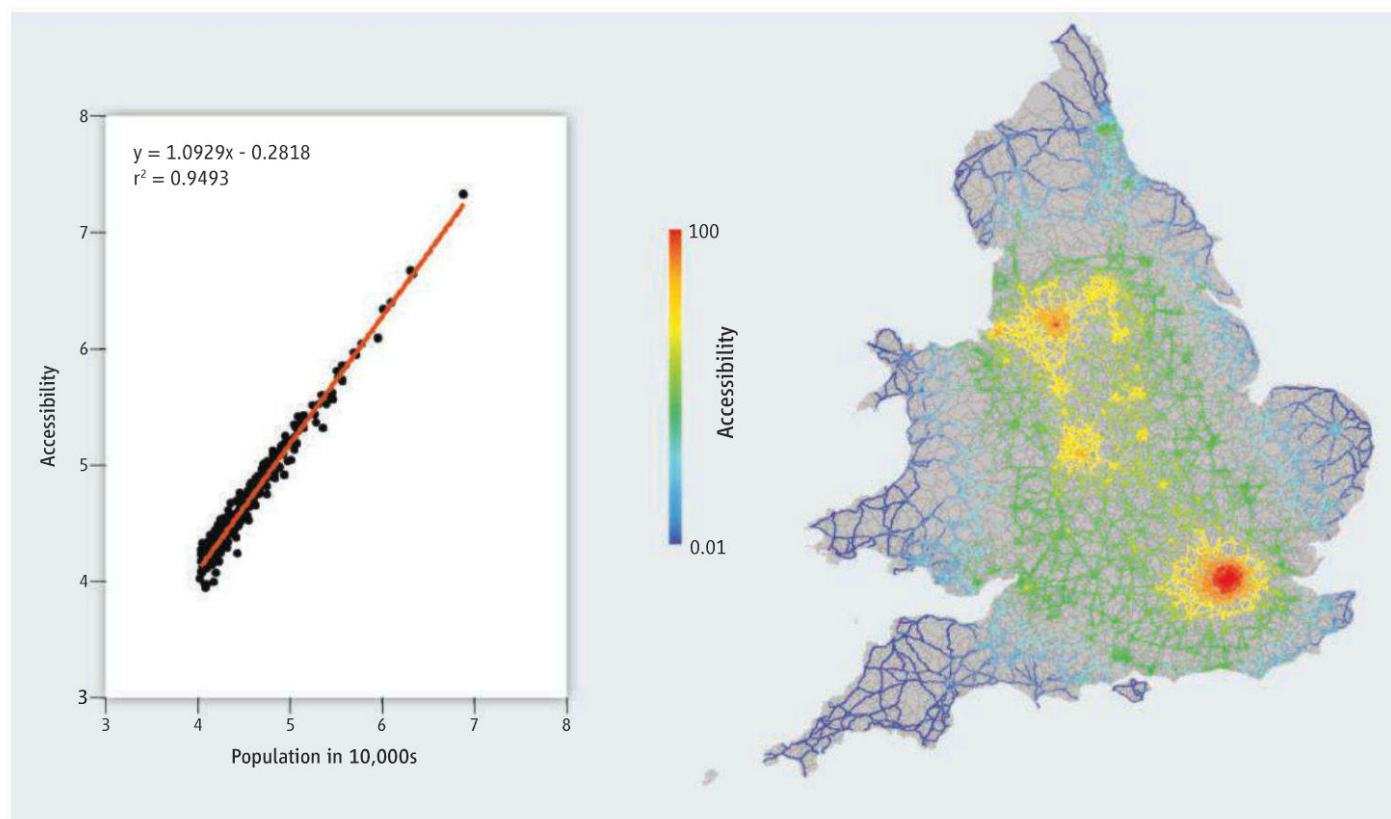
There is substantial evidence for these scaling relationships in countries such as in the United States, where cities are widely spaced and do not generally merge into one another. It is even consistent for countries that have dominant, or so-called single primate cities unrivaled by others, such as London, which tend to be outliers in city systems that do not manifest clear scaling. Bettencourt develops a theory based on a social dynamic that accounts for these observations. His argument turns on associating the trade-off between the positive feedback associated with social interactions generating more than proportionate increases in resources and the negative feedbacks resulting from mixing of populations over ever larger areas as cities get bigger. These negative feedbacks include those associated with travel and congestion.

A theory explaining how the attributes of cities scale with city size may help to inform urban planning.

This simple but appealing and plausible idea resonates with what we know about cities. The power of Bettencourt's analysis is to show how the trade-off between benefits and costs leads to increases in quantities that vary superlinearly with population size, such as income and resources, and infrastructures that vary sublinearly, such as the areas and lengths of physical links such as streets. He disentangles this theory by associating the ways in which we build infrastructures incrementally as treelike networks, showing that his derivations of scaling relations can be related to the underlying fractal geometry of the cities under scrutiny (5).

Bettencourt develops a recursive formulation for this geometry, using analogies with electric circuits to show how energy dissipates and scales like human interactions, with the ratio of income to total power used being a constant with respect to city size. He then reworks this framework using a maximization of the differences between income and energy with respect to average social outputs subject to known constraints. This implies the same kinds of scaling that are introduced in his initial discussion of

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Superlinear scaling of accessibility for cities in England and Wales. Accessibilities to all 8850 small areas (wards) from 283 towns and cities with a population of more than 10,000 are computed over the road network, which is colored according to the accessibility of the nearest town. The inset shows the regression of accessibility on population (10), which extends Bettencourt's theory to embrace spatial economies of scale based on interactions between cities.

the theory, but it produces more detail pertaining to ways in which cities might depart from the generic scaling derived here.

What is particularly elegant about this theory is its quest for explaining scaling in terms of both the fractal and Euclidean geometry of the city, because it is in terms of these geometries that urban planning has its primary impact. It might be argued that to solve the problems of the city, we need something more than intervention in its physical form (for example, changes in local taxation), but physical plans still mark the way in which we might influence the shape and quality of life in our cities in the least intrusive way.

Bettencourt identifies some revealing tensions in cities that suggest how the physical forms that we might influence can be made clearer, particularly with respect to mobility, transport infrastructure, and density. His theory deals with phenomena within cities, albeit with respect to different sizes of free-

standing cities. However, it says little so far about the "system of cities" (6) that dominates the modern era of globalization. Given that all humans are predicted to be living in cities by the end of this century (7), and that it will often be hard to know where one city ends and another begins (as is true already in many parts of the world), it remains to be shown how well this theory of the individual city will hold up.

In fact, all the rudiments for extending these ideas to systems of cities are in place here. Extension to other cities can be inserted into Bettencourt's theory as some measure of flows into and out of one particular city from and/or to all other cities. This flow might be represented as a gravitational potential or accessibility, with the relative nearness of all places of a particular size moderated by the distance or cost of getting to those places (8). It is often hard to define unambiguous boundaries around cities—for instance, in the United Kingdom—but if we use the augmented scaling relation (9) and regress this against population for all 283 towns and cities with populations over 10,000 in England and Wales, then we generate superlinear scaling for the system of cities (see the figure) (10). Many questions result from such extensions, particularly regarding the robustness of the superlinearity that pertains to the scaling.

In the current thinking about cities, the situation is not very different from that implied by Maxwell in the middle of the 19th century when he said that "the dimmed outlines of phenomenal things all merge into one another unless we put on the focussing glass of theory" (11). Bettencourt provides us with the essence of a theory that begins to make sense of how cities change as they scale in a world that is rapidly becoming entirely urbanized.

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Liquid Exfoliation of Layered Materials

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READ THE FULL ARTICLE ONLINE
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Background: Since at least 400 C.E., when the Mayans first used layered clays to make dyes, people have been harnessing the properties of layered materials. This gradually developed into scientific research, leading to the elucidation of the laminar structure of layered materials, detailed understanding of their properties, and eventually experiments to exfoliate or delaminate them into individual, atomically thin nanosheets. This culminated in the discovery of graphene, resulting in a new explosion of interest in two-dimensional materials.

Layered materials consist of two-dimensional platelets weakly stacked to form three-dimensional structures. The archetypal example is graphite, which consists of stacked graphene monolayers. However, there are many others: from MoS_2 and layered clays to more exotic examples such as MoO_3 , GaTe, and Bi_2Se_3 . These materials display a wide range of electronic, optical, mechanical, and electrochemical properties. Over the past decade, a number of methods have been developed to exfoliate layered materials in order to produce monolayer nanosheets. Such exfoliation creates extremely high-aspect-ratio nanosheets with enormous surface area, which are ideal for applications that require surface activity. More importantly, however, the two-dimensional confinement of electrons upon exfoliation leads to unprecedented optical and electrical properties.

Advances: An important advance has been the discovery that layered crystals can be exfoliated in liquids. There are a number of methods to do this that involve oxidation, ion intercalation/exchange, or surface passivation by solvents. However, all result in liquid dispersions containing large quantities of nanosheets. This brings considerable advantages: Liquid exfoliation allows the formation of thin films and composites, is potentially scaleable, and may facilitate processing by using standard technologies such as reel-to-reel manufacturing.

Although much work has focused on liquid exfoliation of graphene, such processes have also been demonstrated for a host of other materials, including MoS_2 and other related structures, layered oxides, and clays. The resultant liquid dispersions have been formed into films, hybrids, and composites for a range of applications.

Outlook: There is little doubt that the main advances are in the future. Multifunctional composites based on metal and polymer matrices will be developed that will result in enhanced mechanical, electrical, and barrier properties. Applications in energy generation and storage will abound, with layered materials appearing as electrodes or active elements in devices such as displays, solar cells, and batteries. Particularly important will be the use of MoS_2 for water splitting and metal oxides as hydrogen evolution catalysts. In addition, two-dimensional materials will find important roles in printed electronics as dielectrics, optoelectronic devices, and transistors.

To achieve this, much needs to be done. Production rates need to be increased dramatically, the degree of exfoliation improved, and methods to control nanosheet properties developed. The range of layered materials that can be exfoliated must be expanded, even as methods for chemical modification must be developed. Success in these areas will lead to a family of materials that will dominate nanomaterials science in the 21st century.

Liquid exfoliation of layered crystals allows the production of suspensions of two-dimensional nanosheets, which can be formed into a range of structures. (A) MoS_2 powder. (B) WS_2 dispersed in surfactant solution. (C) An exfoliated MoS_2 nanosheet. (D) A hybrid material consisting of WS_2 nanosheets embedded in a network of carbon nanotubes.

ARTICLE OUTLINE

Why Exfoliate?

Large-Scale Exfoliation in Liquids?

Pioneers

Recent Advances in Liquid Exfoliation

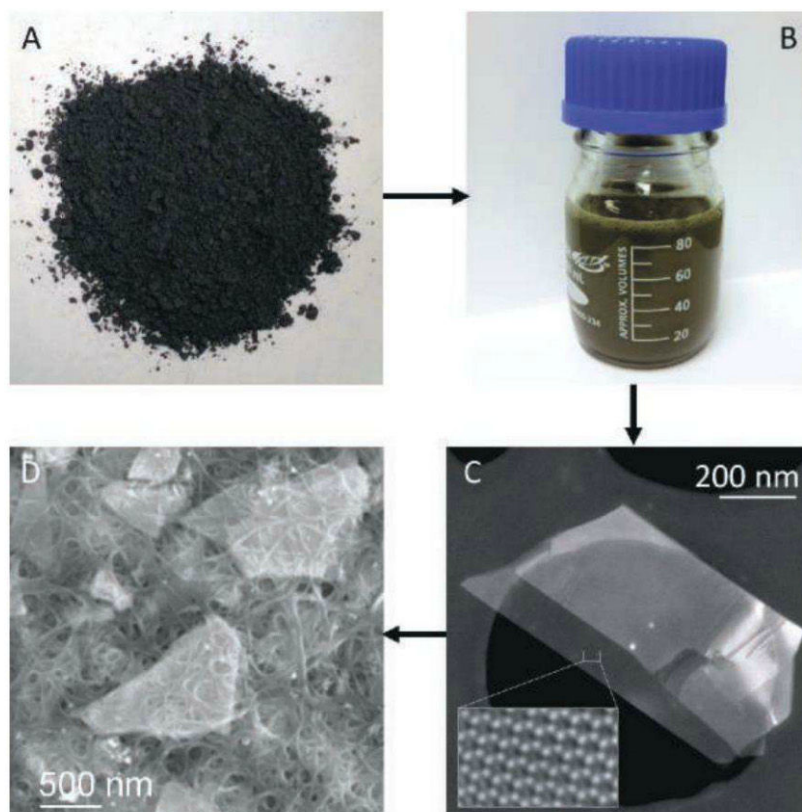
Potential Applications of Liquid-Exfoliated Nanosheets

Outlook

ADDITIONAL RESOURCES

J. N. Coleman *et al.*, Two-dimensional nanosheets produced by liquid exfoliation of layered materials. *Science* **331**, 568–571 (2011). doi:10.1126/science.1194975

K. Varoon *et al.*, Dispersible exfoliated zeolite nanosheets and their application as a selective membrane. *Science* **334**, 72–75 (2011). doi:10.1126/science.1208891



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Liquid Exfoliation of Layered Materials

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Michael S. Strano,⁵ Jonathan N. Coleman^{1*}

Not all crystals form atomic bonds in three dimensions. Layered crystals, for instance, are those that form strong chemical bonds in-plane but display weak out-of-plane bonding. This allows them to be exfoliated into so-called nanosheets, which can be micrometers wide but less than a nanometer thick. Such exfoliation leads to materials with extraordinary values of crystal surface area, in excess of 1000 square meters per gram. This can result in dramatically enhanced surface activity, leading to important applications, such as electrodes in supercapacitors or batteries. Another result of exfoliation is quantum confinement of electrons in two dimensions, transforming the electron band structure to yield new types of electronic and magnetic materials. Exfoliated materials also have a range of applications in composites as molecularly thin barriers or as reinforcing or conductive fillers. Here, we review exfoliation—especially in the liquid phase—as a transformative process in material science, yielding new and exotic materials, which are radically different from their bulk, layered counterparts.

In 1824, Thomas H. Webb heated a mineral similar to mica and, by means of thermal exfoliation, transformed it into what is today a valuable commodity, with applications as an ion exchange resin, an insulating material, and a structural binder in cement. He named the mineral “vermiculite” for its wormlike appearance upon exfoliation (Fig. 1), from the Latin *vermiculare* meaning “to breed worms.” Almost 200 years later, in 2004, Geim and Novosolov showed that thin transparent adhesive tape could be used to exfoliate graphite into single atomic layers of graphene and demonstrated atomically thin devices (*1*). As a process, exfoliation of layered solids has had a transformative effect on materials science and technology by opening up properties found in the two-dimensional (2D) exfoliated forms, not necessarily seen in their bulk counterparts.

Layered materials are defined as solids with strong in-plane chemical bonds but weak out-of-plane, van der Waals bonds. Such materials can be sheared parallel or expanded normal to the in-plane direction. In the extreme limit, these processes yield nanometer-thin—even atomically thin—sheets that are not at all characteristic of the bulk precursor. This production of extremely thin sheets from layered precursors is known as exfoliation or delamination, although in this work we will use the former term. The sheets produced are generally referred to as nanosheets, where “nano” refers to the magnitude of the thickness. Although in the ideal case such nanosheets consist of single monolayers, they are often man-

ifested as incompletely exfoliated flakes comprising a small number (<10) of stacked monolayers.

There are many types of layered materials, which can be grouped into diverse families (Fig. 1). The simplest are the atomically thin, hexagonal sheets of graphene (*1–3*) and hexagonal boron nitride (h-BN) (*4*). Transition metal dichalcogenides (TMDs) (such as MoS₂ and WSe₂) (*5, 6*) and metal halides (such as PbI₂ and MgBr₂) (*7*) have near-identical structures and consist of a plane of metal atoms sandwiched between planes of halide/chalcogen atoms. Layered metal oxides (such as MnO₂, MoO₃, and LaNb₂O₇) (*8–11*) and layered double hydroxides (LDHs) [such as Mg₆Al₂(OH)₁₆] (*8, 12*) represent a diverse class of materials with a large variety of structures. Similarly, layered silicates, or clays, are minerals and exist as many different types, with well-known examples being montmorillonite or the micas (*13, 14*). Generally, oxides, LDH, and clay nanosheets are charged and are accompanied by charge-balancing ions (*8, 14*). Other interesting families are the layered III-VIs (such as InSe and GaS) (*15*), the layered V-VIs (such as Bi₂Te₃ and Sb₂Se₃) (*16*), the metal trichalcogenides, and metal trihalides. Although many other layered materials exist (Table 1), all share a planar, anisotropic bonding and therefore the potential to be exfoliated into nanosheets.

One substantial advantage of layered materials is their diversity. Even before exfoliation, the many families of layered materials display a very broad spectrum of properties. For example, TMDs (*5, 6*) occur as more than 40 different types depending on the combination of chalcogen (S, Se, or Te) and transition metal (*5, 6*). Depending on the coordination and oxidation state of the metal atoms, or doping of the lattice, TMDs can be metallic, semimetallic, or semiconducting (*6*). In addition, these materials display interesting electronic behavior, such as superconductivity or charge-density wave effects (*6*). Similarly, the many different types of layered metal oxides have interesting

electronic, electrochemical, and photonic properties (*8*). These materials have been fabricated into transistors, battery electrodes, and magneto-optic devices (*8–10*). Thus, even as bulk crystals, layered materials are an interesting and potentially useful material class. This makes them an exciting starting material for exfoliation into nanosheets. As we will see below, exfoliation dramatically enhances the range of properties displayed by an already diverse material type.

Why Exfoliate?

The simplest effect of exfoliation is to dramatically increase the accessible surface area of a material. For surface-active or catalytic materials, this can radically enhance their chemical and physical reactivity. The ion exchange ability of minerals such as vermiculite to purify water at 1000 meq/kg depends on its near 10⁶-fold increase in surface area after expansion (*13*). In structural mechanics, the strength and stiffness of composites increase as the thickness of planar fillers, such as clay or graphite, decreases (*17*). When heat causes exfoliation, a layered material can be used as an intumescent (or thermally expansive) material. Hence, vermiculite and graphite are used for fire retardation in paints and firestop pillows because they reduce their density upon heating and produce an ash of low thermal conductivity.

As interest in nanotechnology has intensified in recent decades, another important advantage of exfoliation has emerged. In a layered crystal, the electronic wave function extends in three dimensions. However, after exfoliation electrons are constrained to adopt a 2D wave function, thus modifying the electronic band structure. Graphite can be transformed into a graphene monolayer after exfoliation, with electronic properties that differ greatly from any other material (*1*). These include an enormously high carrier mobility and other exciting properties, such as Klein tunnelling and the half-integer quantum Hall effect (*1, 3*). Likewise, the properties of MoS₂ depend strongly on exfoliation state. The bandgap of MoS₂ changes on exfoliation from 1.3 eV for the bulk crystal to 1.9 eV for an exfoliated nanosheet. Because the bandgap changes monotonically with number of monolayers per nanosheet, this allows the electronic response to be chosen at will (*18*). In addition, although multilayer MoS₂ is not photoluminescent, exfoliation-induced changes in its electronic structure lead to photoluminescent behavior in exfoliated monolayers (*19*). Similar behavior is expected in other layered semiconductors (*5*).

Large-Scale Exfoliation in Liquids?

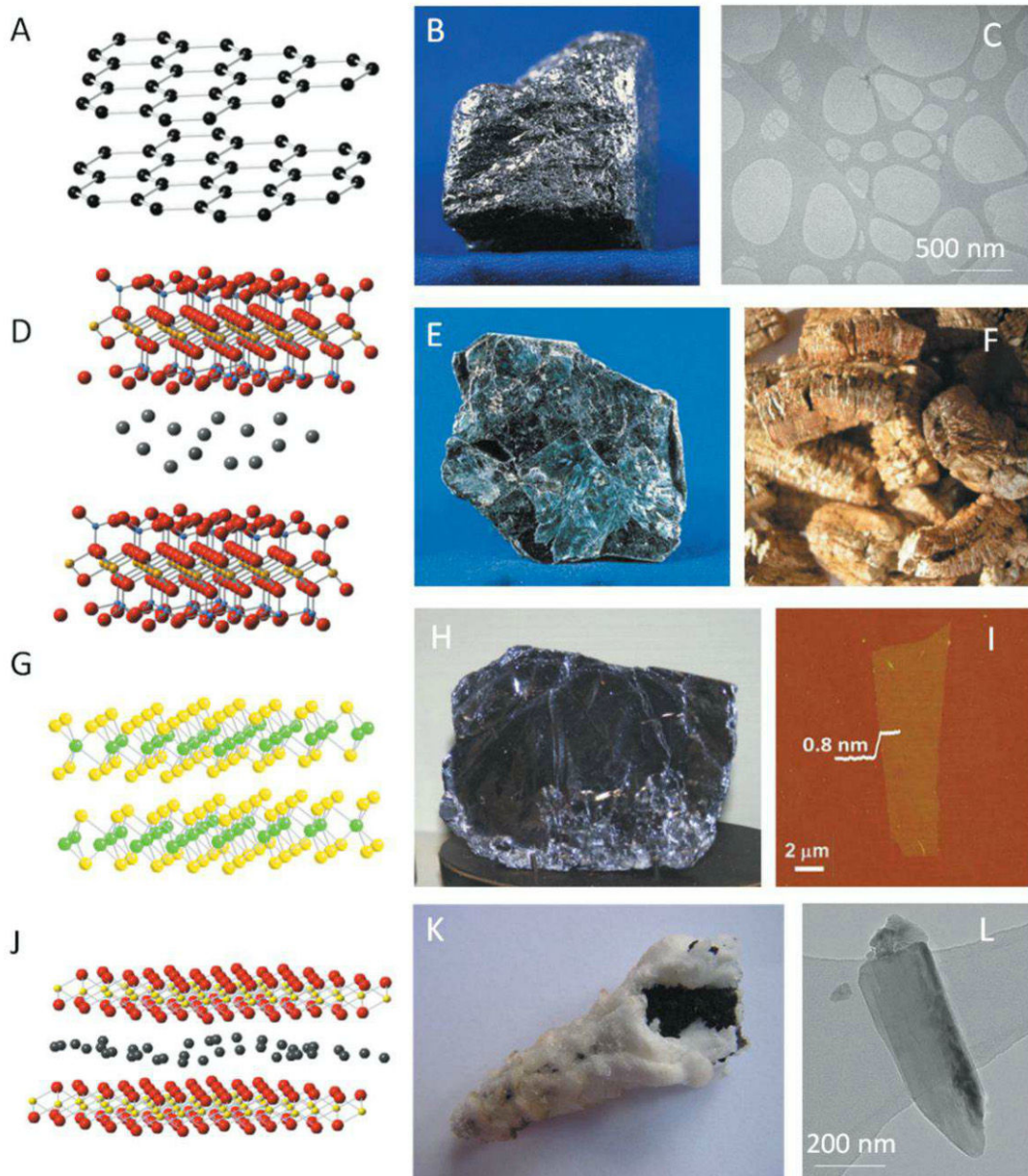
The exfoliation of graphite demonstrated by Geim and Novosolov was achieved essentially by rubbing graphite on a surface (*1*). Such mechanical exfoliation remains the source of the highest-quality graphene samples available and has resulted in some major advances (*1*). However, it suffers from low yield and a production rate that is not

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Fig. 1. Crystal structures, naturally occurring forms, and exfoliated products for four example layered materials.

(A) Graphite consists of alternating stacks of hexagonally arranged carbon atoms (black spheres), (B) is a naturally occurring mineral, and (C) exfoliates to single atomic layers of carbon called graphene. (D) Vermiculite is a layered silicate hydrate (typically $\text{Mg}_{1.8}\text{Fe}_{0.9}\text{Al}_{4.3}\text{SiO}_{10}(\text{OH})_2 \cdot 4(\text{H}_2\text{O})$) that (E) is found naturally as a mineral and (F) can be exfoliated, for example, upon heating. Silicon atoms are in blue, oxygen atoms are in red, Al/Mg/Fe atoms are in yellow, and interlayer counterions are in black (H and H_2O not shown). (G) MoS_2 is a layered arrangement of S and Mo atoms (chalcogen atoms are in yellow, and transition metal are in green) that (H) is found naturally as the mineral molybdenite and (I) can be exfoliated to MoS_2 monolayers. (J) Layered manganese dioxide (manganese atoms are in yellow, oxygen is in red, and interlayer counterions are in black) occurs naturally (K) as birnessite and (L) can be exfoliated to give MnO_2 nanosheets. (C), (I), and (L) are adapted from (48), (87), and (58), respectively. The layer spacings for each material are graphite, 0.35 nm; vermiculite, 1.5 nm; MoS_2 , 0.6 nm; and MnO_2 , 0.45 nm.



technologically scalable in its current form. One possible solution is the exfoliation of layered compounds in liquids to give large quantities of dispersed nanosheets. This should allow for methods to obtain sizable quantities of 2D materials that can be processed by using existing industrial techniques, such as reel-to-reel manufacturing. Here, we briefly outline the four main liquid exfoliation techniques for layered materials (schematics are provided in Fig. 2, and examples of exfoliated nanosheets are provided in Fig. 3).

One of the oldest methods of exfoliating layered crystals with low reductive potential is oxidation and subsequent dispersion into suitable solvents. The best example is that of graphite (20), in which treatment with oxidizers such as sulphuric acid and potassium permanganate results in addition of hydroxyl and epoxide groups to the

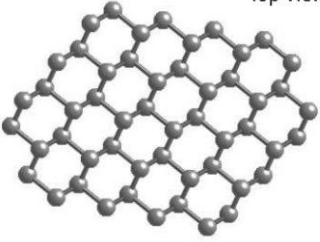
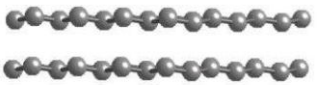
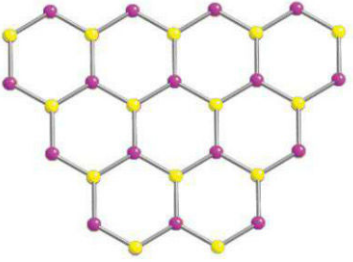
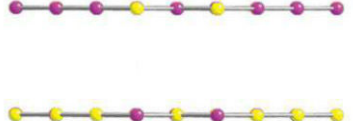
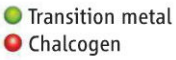
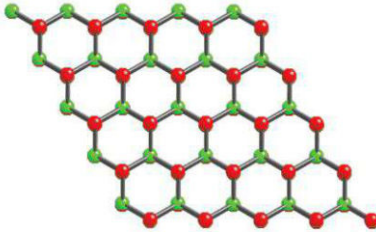
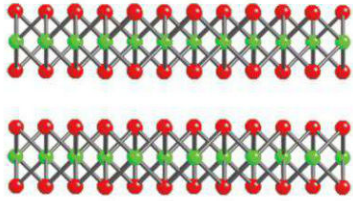
basal plane. The resulting hydrophilicity allows water intercalation and large-scale exfoliation to yield graphene oxide upon ultrasonication. The dispersed flakes are predominantly monolayers, typically hundreds of nanometers across, and stabilized against reaggregation by a negative surface charge at concentrations of up to 1 mg/ml. Dispersed graphene oxide can be chemically reduced in the liquid phase but will then aggregate unless surfactant or polymer stabilizers are present. Although reduction removes most of the oxides, structural defects remain, rendering the properties of oxidatively produced graphene substantially different from pristine graphene.

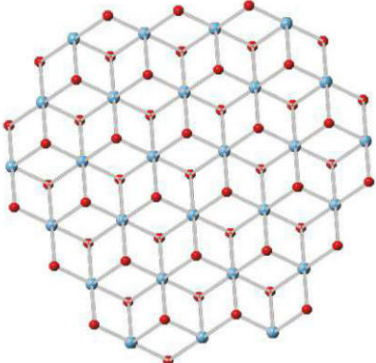
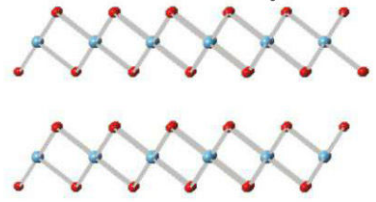
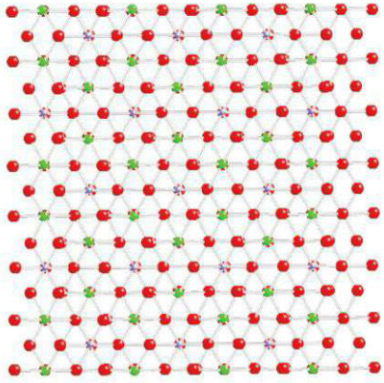
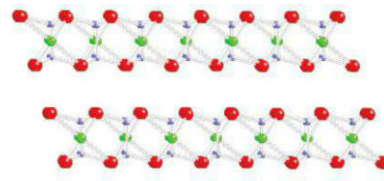
Layered materials can also strongly adsorb guest molecules into the spacing between layers, creating what are called inclusion complexes. This forms the basis of another exfoliation method that is widely applied to layered materials,

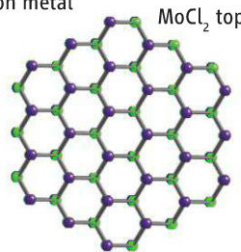
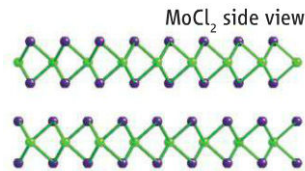
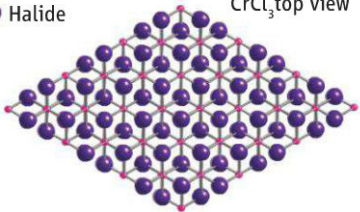
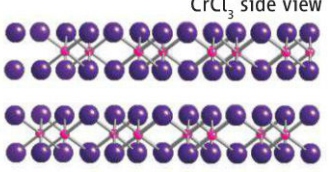
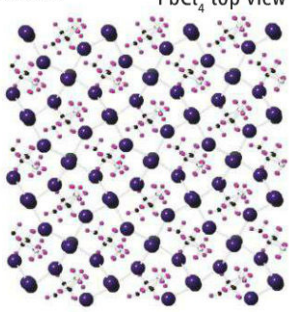
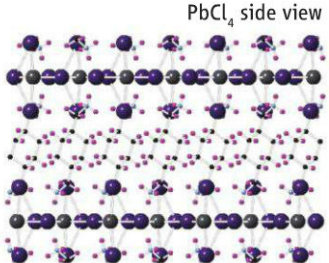
including graphite (21) and TMDs (22, 23). Intercalation, often of ionic species, increases the layer spacing, weakening the interlayer adhesion and reducing the energy barrier to exfoliation. Intercalants such as *n*-butyllithium (22, 23) or IBr (21) can transfer charge to the layers, resulting in a further reduction of interlayer binding. Subsequent treatment such as thermal shock (21) or ultrasonication (22, 23) in a liquid completes the exfoliation process. The exfoliated nanosheets can be stabilized electrostatically by a surface charge (23) or by surfactant addition (21). In the case of MoS_2 , this method tends to give highly exfoliated nanosheets (22). However, ion intercalation-based methods have drawbacks associated with their sensitivity to ambient conditions (22–24).

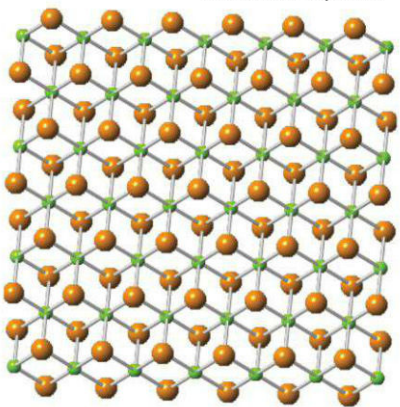
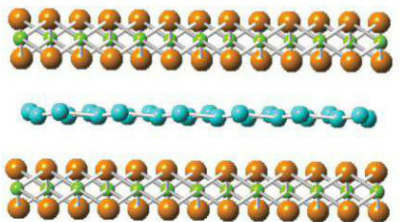
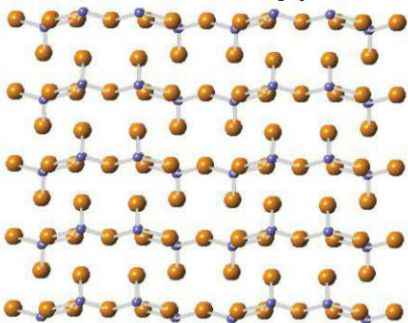
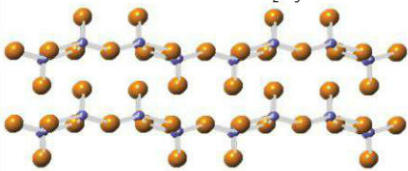
Ion exchange methods take advantage of the fact that LDHs, clays, and some metal oxides

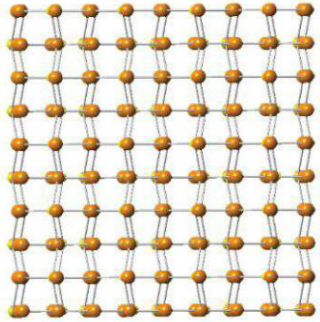
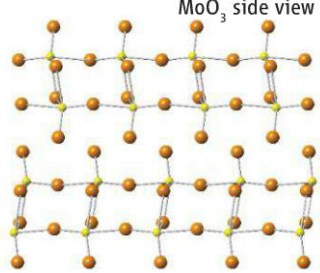
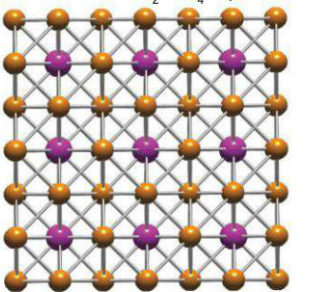
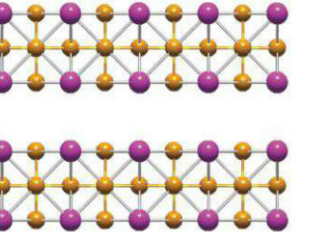
Table 1. Referenced table of families of layered compounds, including structures and information on exfoliation methods, potential applications, and availability. This table is not exhaustive. Crystal structures were obtained from the CrystalMaker Library (www.crystallmaker.com/library/index.html).

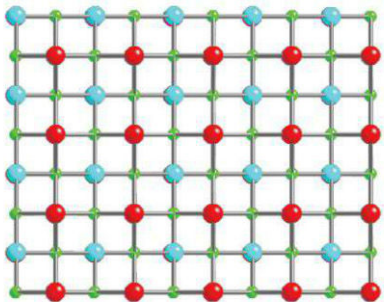
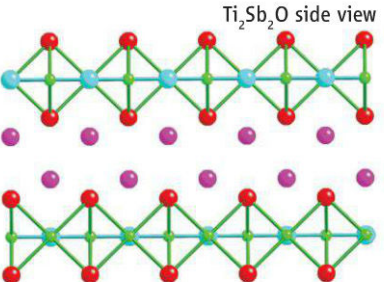
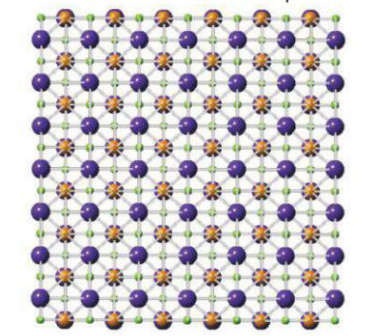
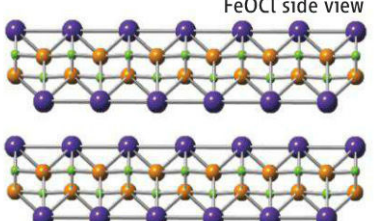
Family of layered compound	Structure	Exfoliation method	Applications	Commercial availability
Graphite	Top view  Side view 	Sonication in surfactant solution (30, 50–53) Sonication in solvents (27, 45–48) Sonication in polymer solutions (54, 55) Graphene oxide (20, 88)	Many (1, 89)	Widely available
h-BN	 Top view  Side view 	Sonication in surfactant solution (58) Sonication in solvents (29, 56) Sonication in polymer solutions (54)	Composites (57) Device substrates (90)	Yes
Transition metal dichalcogenides (TMDs)	 MoS₂ top view  MoS₂ side view 	Sonication in surfactant solution (58) Sonication in solvents (29, 59, 60) Sonication in polymer solutions (54) Ion intercalation (91)	Single-layer transistor (92) Batteries (63, 64) Top-gate phototransistors (93) Thermo-electrics (29, 58) Superconducting composites (94)	Raw materials mostly available (purity issues)

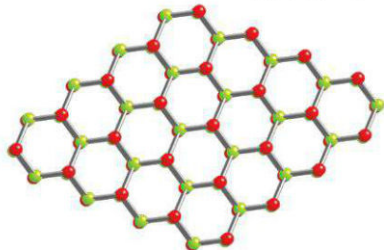
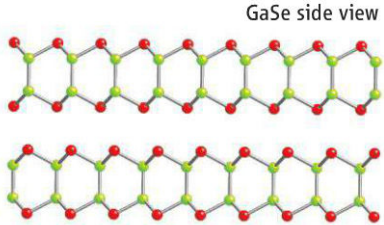
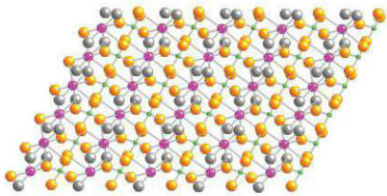
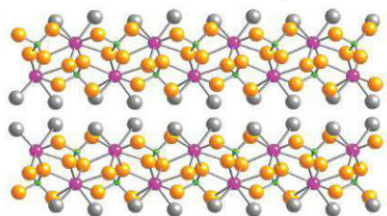
Transition metal trichalcogenides (TMDs)	AMo_3X_3, NbX_3, TiX_3, and TaX_3 ($\text{X} = \text{S}$, Se, or Te) ● Transition metal ● Chalcogen TiTe_3 top view  TiTe_3 side view 	Ion intercalation (95)	Batteries (96)	No, only by synthesis
	Metal phosphorous trichalcogenides (MPX_3), such as MnPS_3, CdPS_3, NiPS_3, ZnPS_3, and $\text{Mn}_{0.5}\text{Fe}_{0.5}\text{PS}_3$ ● Transition metal ● Chalcogen ● Phosphorus MnPS_3 top view  MnPS_3 side view 	Intercalation (75)	Wide band-gap semiconductors (97) Magnetic properties (98)	No, only by synthesis

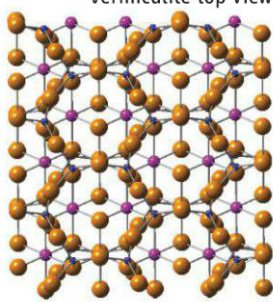
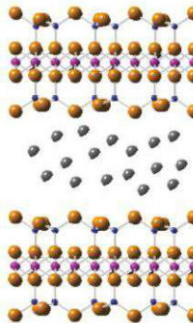
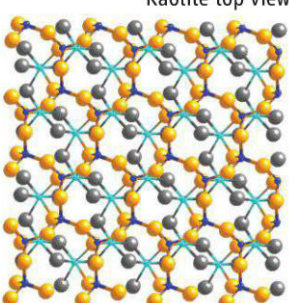
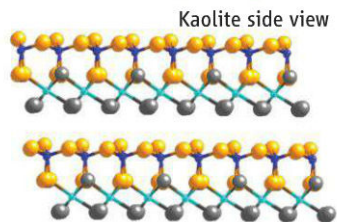
Metal halides	Transition-metal dihalides* ● Transition metal ● Halide MoCl₂ top view  MoCl₂ side view 	Ion intercalation (7) Polymer intercalation (99)		No (synthesis required)
	Metal MX₃ halides, such as αRuCl₃, CrCl₃, and BiI₃† ● Transition metal ● Halide CrCl₃ top view  CrCl₃ side view 	Ion intercalation (100) Polymer intercalation (99)		No (synthesis required)
	Layer-type halides with composition MX₄, MX₅, MX₆‡ ● Heavy metal ● Halide ● Ammonium PbCl₄ top view  PbCl₄ side view 	Ion intercalation (101)		No (synthesis required)

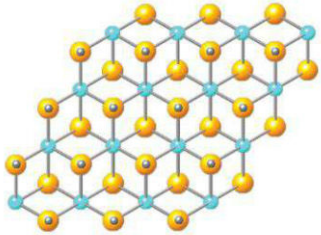
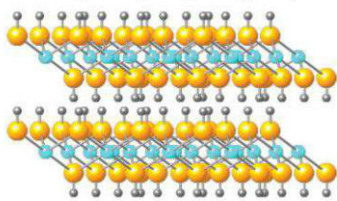
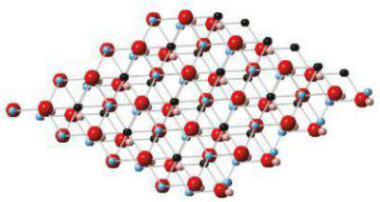
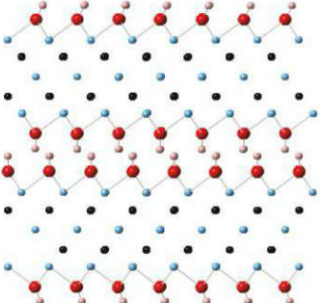
Oxides	Transition metal oxides : Ti oxides, $\text{Ti}_{0.91}\text{O}_2$, $\text{Ti}_{0.87}\text{O}_2$, Ti_3O_7, Ti_4O_9, Ti_5O_{11}; Nb oxides, Nb_3O_8, Nb_6O_{17}, HNb_3O_8; Mn oxides, MnO_2, Ti_3O_7, $\text{Na}_x(\text{Mn}^{4+}, \text{Mn}^{3+})_2\text{O}_4$ ● Transition metal ● Oxygen ● Cation $\text{Na}_x(\text{Mn}^{4+}, \text{Mn}^{3+})_2\text{O}_4$ (birnessite) top view  $\text{Na}_x(\text{Mn}^{4+}, \text{Mn}^{3+})_2\text{O}_4$ (birnessite) side view  ● Vanadium ● Oxygen Vanadium oxide (V_2O_5) top view  V_2O_5 side view 	Ti oxides Ion intercalation (102) Mn oxides Sonication in surfactant solution (58); Ion intercalation (103) Nb oxides Ion intercalation (104) Va oxides Polymer intercalation (105)	Supercapacitors (106) Batteries (107) Catalysts (108) Dielectrics (109) Ferroelectrics (109)	Some raw materials available (purity issues) Most compounds are not available
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Oxides	Trioxides, such as MoO_3, TaO_3, and hydrated WO_3 Hydrogen Oxygen Transition metal MoO_3 top view  MoO_3 side view 	Ion intercalation (110) Polymer intercalation (111)	Electrochromics (112) Light emitting diodes (113)	Yes
	Layered trirutile phases HMWO ($M = \text{Nb}, \text{Ta}$), such as (HNbWO_6 and HTaWO_6)	Ion intercalation (protonation and ion exchange) (114)		No (only by synthesis)
	Perovskites and niobates, such as Sr_2RuO_4, $\text{KCa}_2\text{Nb}_3\text{O}_{10}$, $\text{H}_2\text{W}_2\text{O}_7$, LaNb_2O_7, $\text{La}_{0.90}\text{Eu}_{0.05}\text{Nb}_2\text{O}_7$, $\text{Eu}_{0.56}\text{Ta}_2\text{O}_7$, Sr_2RuO_4, $\text{Sr}_3\text{Ru}_2\text{O}_7$, SrTa_2O_7, $\text{Bi}_2\text{SrTa}_2\text{O}_9$, $\text{Ca}_2\text{Nb}_3\text{O}_{10}$, $\text{Sr}_2\text{Nb}_3\text{O}_{10}$, $\text{NaCaTa}_3\text{O}_{10}$, $\text{CaLaNb}_2\text{TiO}_{10}$, $\text{La}_2\text{Ti}_2\text{NbO}_{10}$, and $\text{Ba}_5\text{Ta}_4\text{O}_{15}$ Strontium Ruthenium Oxygen Sr_2RuO_4 top view  Sr_2RuO_4 side view 	Intercalation with liquid crystals (115) Ion intercalation (protonation and ion exchange) (116) Amine surfactant (TBA^+) under sonication (117)	Ferroelectrics (118) Photochromic (119) Photoluminescent (120)	No (only by synthesis)

Oxides	Oxychalcogenides and oxypnictides: Oxychalcogenides, LaOCuCh (Ch, chalcogenide) and derivatives, $\text{Sr}_2\text{MO}_2\text{Cu}_{2-5}\text{S}_2$ ($M = \text{Mn, Co, Ni}$), $\text{Sr}_2\text{MnO}_2\text{Cu}_{2m-0.5}\text{S}_{m+1}$ ($m = 1-3$), $\text{Sr}_4\text{Mn}_3\text{O}_{7.5}$, Cu_2Ch_2 ($\text{Ch}=\text{S, Se}$); oxypnictides, LaOFeAs ● Transition metal ● Pnictide ● Cation $\text{Ti}_2\text{Sb}_2\text{O}$ top view  $\text{Ti}_2\text{Sb}_2\text{O}$ side view 	To our knowledge, these have never been exfoliated	Superconductivity (121) Magnetic properties (121) Catalyst (redox properties) (121) Batteries (121)	No (only by synthesis)
	Oxyhalides of transition metals, such as VOCl, CrOCl, FeOCl, NbO_2F, WO_2Cl_2, and FeMoO_4Cl ● Transition metal ● Halide ● Oxygen FeOCl top view  FeOCl side view 	Ion intercalation (122)	Batteries (123)	No (only by synthesis)

III–VI layered semiconductor	GaX (X = S, Se, Te); InX (X = S, Se, Te)[¶] ● S/Se/Te ● Ga/In GaSe top view  GaSe side view 	Ion intercalation (124) Surfactant (125)	Nonlinear optical properties, poor thermal conductivity (126)	Some available (maybe purity issues), mostly synthesized
Layered α and γ zirconium phosphates and phosphonates	α-M^{IV} phosphates, α-M^{IV}(O₃P–OH)₂·H₂O; and α-Metal^{IV} phosphonates, M^{IV}(O₃P–R)₂·nH₂O# ● Oxygen ● Phosphorus ● Cation ● Water CaHPO₄ top view  CaHPO₄ side view 	Intercalation (127) Exfoliation in water/acetone mixtures (128)	Drug delivery (127) Semiconductor for dye sensitized solar cells (129)	No (only by synthesis)

Clays (layered silicates)	2:1 Layered silicates**: Smectites, $M^{n+}_{x/n} \cdot yH_2O[Al_{4-x}Mg_x](Si_8)O_{20}(OH)_4$; talc, $[(Mg_3)(Si_2O_5)_2(OH)_2]$; vermiculite, $[Mg_6Si_6Al_2O_{20}(OH)_4][M^{n+}_{1/n}](Mg_6)$; biotite $[(MgFe)_3(Si_3Al)O_{10}(OH)_2]K$; phlogopite, $[(Mg_6)(Si_6Al_2)O_{20}(OH)_4]Ba$; fluorophlogopite, $[Mg_{11/4}(Si_6Al_2)O_{20}F_4][M^{2+}_{3/2}]$; margarite, $[(Al_2)(Si_2Al_2)O_{10}(OH)_2]Ca$; and muscovite, $[(Al_2)(Si_3Al)O_{10}(OH)_2]K$ ● Oxygen ● Cation ● Silicon ● Intercalates/-C/-OH Vermiculite top view  Vermiculite side view  1:1 Layered Silicates ††: Kaolinite, $[Al_4Si_4O_{10}](OH)_8$; halloysite, $Al_4Si_4O_{10}(OH)_8 \cdot 4H_2O$ ● Aluminum ● Silicon ● Oxygen ● OH- Kaolinite top view  Kaolinite side view 	Dispersion in water (13) Intercalation (130) Polymer intercalation (131)	Catalysis (130) Composites (131) Lightweight nanocomposites for structural applications (132) Clay-dye complexes and photoactive materials (131) Organoclays as ionic and electronic conductors (composites with conductive polymers) (131) Thermal and barrier properties nanocomposites (133)	Natural minerals, available on the market
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Layered double hydroxides (LDHs)	General formula: $M(II)_{1-x}M(III)_x(OH)_2(A^{n-})_{x/n} \cdot yH_2O$, where $M(II)$ = divalent cation; $M(III)$ = trivalent cation; A = interlayer anion; and n = charge on interlayer anion$\dagger\dagger$ ● Oxygen ● Cation ● Hydrogen Brucite ($Mg^{2+}_x, Mg^{3+}_x(OH)_2(A^{n-})_{x/n} \cdot yH_2O$) top view  Brucite ($Mg^{2+}_x, Mg^{3+}_x(OH)_2(A^{n-})_{x/n} \cdot yH_2O$) side view 	Intercalation (134) Surfactant-assisted exfoliation and intercalation of molecules (135) Surfactant exfoliation (136) Solvent exfoliation in DMF (137) Functionalization followed by exfoliation in solvents (138)	Biocompatible–bio-hybrids/drug delivery (12, 134) Bionanocomposites with functional and structural properties (139)	No, only by synthesis
Ternary transition metal carbides and nitrides	Derivatives from MAX phases, where M = transition metal; A = Al or Si; and X = C or N$\S\S$ ● Carbon ● Oxygen ● Titanium ● Hydrogen  Ti3C2 	Extra oxygen and hydrogen at layers surface are present as a consequence of the exfoliation treatment with HF (66, 67).	Batteries and supercapacitors (140)	Yes

*These are iso-structural with TMDs. $\dagger$ These are defect CdI₂ structure types. $\ddagger$ These are heavy metal halides (perovskite type) structurally similar to transition metal dihalides with organic ammonium interlayers. $\S$ Protons emplaced between 2D of $Nb_3O_8^-$ anion nanosheets composed of NbO_6 octahedra. $\parallel$ They contain oxide layers separated by distinct layers, which contain the softer chalcogenide (S, Se, and Te) or pnictide (P, As, Sb, and Bi). $\P$ The building block has a trigonal structure, consisting of a pair of (M_3X_3) rings linked by $M-M$ bonds. Layers interact through van der Waals forces between the X outermost planes. $\#R$ is an organic radical, and n is the number of water molecules that can be intercalated in the interlayer region. $**$ The 2:1 notation means that the layers consist of two tetrahedral silicate sheets sandwiching one octahedral sheet. $\dagger\dagger$ Layer consists of one tetrahedral silicate sheets and one octahedral sheet. $\dagger\dagger\dagger$ The structure of LDHs can be described by considering $Mg(OH)_2$, which consists of Mg^{2+} ions coordinated octahedrally by hydroxyl groups. The octahedral units share edges to form infinite, charge neutral layers. In an LDH, isomorphous replacement of a fraction of the Mg^{2+} ions with a trivalent cation, such as Al^{3+} , occurs and generates a positive charge on the layers that necessitates the presence of interlayer, charge-balancing, anions. The remaining free space of the interlayer is occupied by water of crystallization. $\S\S$ Layered M_2X , M_3X_2 , M_4X_3 , where M = transition metal and X = C or N, can be obtained after removal of the A layer with hydrofluoric acid (HF).

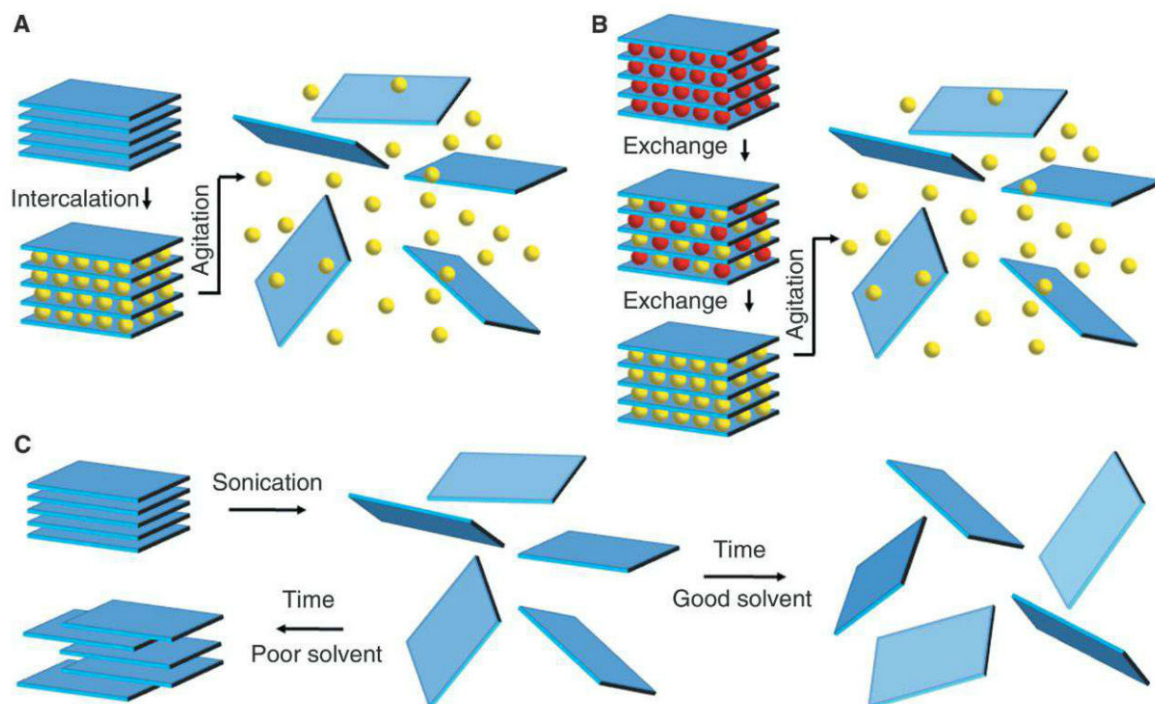


Fig. 2. Schematic description of the main liquid exfoliation mechanisms. (A) Ion intercalation. Ions (yellow spheres) are intercalated between the layers in a liquid environment, swelling the crystal and weakening the interlayer attraction. Then, agitation (such as shear, ultrasonication, or thermal) can completely separate the layers, resulting in an exfoliated dispersion. (B) Ion exchange. Some layered compounds contain ions between the layers so as to balance surface charge on the layers. These ions (red spheres) can be exchanged in a liquid environment for other, often larger ions (yellow

spheres). As above, agitation results in an exfoliated dispersion. (C) Sonication-assisted exfoliation. The layered crystal is sonicated in a solvent, resulting in exfoliation and nanosheet formation. In “good” solvents—those with appropriate surface energy—the exfoliated nanosheets are stabilized against reaggregation. Otherwise, for “bad” solvents reaggregation and sedimentation will occur. This mechanism also describes the dispersion of graphene oxide in polar solvents, such as water. NB, solvent molecules are not shown in this figure.

contain an exchangeable interlayer of cationic counterions (8). For example, titanium dioxide (TiO_2) layered crystals tend to be negatively charged (because of the presence of both Ti^{3+} and Ti^{4+} ions) and so contain counter-ions such as Cs^+ between the layers to ensure charge neutrality (8, 25). Such ions can be exchanged for protons by soaking in acidic solutions. The protons can then be exchanged for bulky organic ions (for example, tetrabutylammonium cations), leading to substantial swelling. Alternatively, some clays containing small monovalent ions such as sodium swell from intercalation of water (13). In general, swelling facilitates exfoliation through ultrasonication or shear mixing to give negatively charged nanosheets (26).

A more recent strategy for exfoliation is to expose the layered material to ultrasonic waves in a solvent (27). Such waves generate cavitation bubbles that collapse into high-energy jets, breaking up the layered crystallites and producing exfoliated nanosheets. Modeling has shown that if the surface energy of the solvent is similar to that of the layered material, the energy difference between the exfoliated and reaggregated states will be very small, removing the driving force for re-aggregation (28). Graphene, h-BN, TMDs, and some TMOs have been exfoliated in this way by using solvents such as *N*-methyl-pyrrolidone (28, 29). Similarly, ultrasonication in surfactant or polymer

solutions gives nanosheets that are electrostatically or sterically stabilized (17, 30).

Pioneers

The exfoliation of layered compounds is an area of research that dates back several centuries. Modern research probably began with Webb and progressed via the work of chemists such as Schafhaeutil, Brodie, and Staudenmaier on the production of graphite intercalation compounds and graphite oxide in the mid-19th century (20). Although these early researchers knew of the laminar nature of graphite, the detailed structure was not known until the 1920s (28). Early attempts to understand exfoliated layered compounds used transmission electron microscopy (TEM) to image few- and possibly even mono-layer exfoliated graphene oxide as early as 1948. However, the limitations of available instrumentation made it impossible to verify whether monolayers were really observed (20, 31). The 1960s saw a flurry of activity on the cleavage (often using adhesive tape) of TMD crystals to produce nanosheets that approached monolayer thickness (32). This was complemented in 1967 by the demonstration that inorganic layered compounds—in this case, vermiculite clay—could be exfoliated in liquids by means of ion intercalation followed by shear mixing (26). In 1975, a similar technique was used to exfoliate TaS_2 , possibly producing monolayers (33). Subsequently, the availability of cheap

ultrasonic agitators provided a more effective energy source for exfoliation, leading to the demonstration of ion intercalation-assisted exfoliation of TaS_2 , NbS_2 , and MoS_2 , as well as layered oxides (23, 34, 35). In these reports, TEM and x-ray diffraction suggested that monolayers had been produced. A decade later in 2000, the exfoliation of layered double hydroxides by means of ion exchange followed by stirring or reflux was demonstrated (36).

Much of this work has historically been limited by the instrumentation available. Early TEM characterization used techniques such as intensity analysis (34) or shadowing (32), which would not meet today's standards of proof for imaging a single layer. However, the development of scanning tunnelling microscopy (STM) and atomic force microscopy (AFM) in the 1980s and recent advances in scanning TEM have changed this entirely. By the time Geim *et al.* produced nanosheets through mechanical exfoliation (1), a new generation of scientific instrumentation with atomic-scale resolution had emerged in order to fully analyze exfoliated materials with unprecedented precision. The result has been a renewed surge in interest in these exotic, molecular-scale materials.

Recent Advances in Liquid Exfoliation

Although the exfoliation of layered materials has a long history, it was interest in graphene that

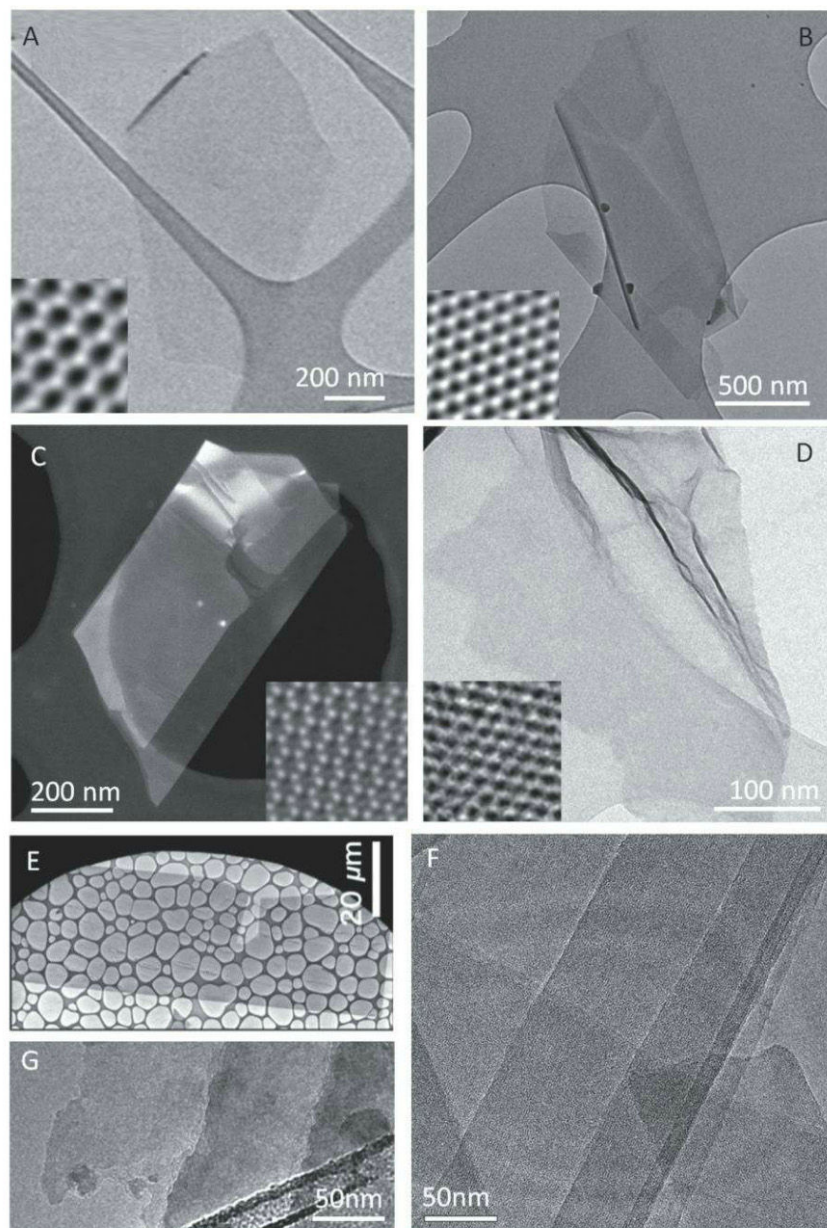


Fig. 3. TEM images of liquid exfoliated nanosheets. (A) A graphene nanosheet exfoliated by means of sonication in the solvent *N*-methyl-pyrrolidone (27). (B) A h-BN nanosheet exfoliated by means of sonication in the solvent isopropanol (29). (C) An MoS₂ nanosheet exfoliated by means of sonication in an aqueous surfactant solution (58). (D) An MoS₂ nanosheet exfoliated by means of Li ion intercalation (22). (E) A TiO₂ nanosheet exfoliated by means of ion exchange (25). (F) Functionalized layered double hydroxide nanosheets exfoliated by means of sonication in ethanol (65). (G) Hydroxylated metal carbide nanosheets exfoliated by means of sonication in methanol (66). Where available, high-resolution images have been included as insets. All images were adapted from the sources as referenced.

stimulated renewed curiosity in this area. For this reason, we outline the recent developments in the liquid exfoliation of layered crystals, using Geim and Novoselov's 2004 paper describing the properties of exfoliated graphene as a starting point.

In a series of papers beginning in 2004 (1, 3, 37, 38), Geim, Novoselov, and collaborators showed that monolayers of graphene and other 2D materials could be removed from their parent crystals by means of mechanical exfoliation and placed on substrates of choice. This was some-

what surprising because theoretical studies had suggested that 2D materials should be intrinsically unstable after exfoliation (2). This apparent contradiction was later resolved with the suggestion that the exfoliated monolayers are stabilized by the formation of ripples that effectively extend the 2D material into the third dimension (39). However, the most exciting result of exfoliation was the demonstration that graphene displayed electronic, mechanical, and thermal properties not seen in any other material. For example, in

addition to the previously undiscovered electronic properties described above, monolayer graphene displays the highest strength and the highest thermal conductivity ever recorded (1–3). Very soon, it became clear that such a material would be required in much larger quantities than mechanical cleavage could deliver. It was immediately obvious that such scalable production might be achieved through liquid exfoliation (40).

One of the first approaches to liquid exfoliation was the oxidation of graphite. In 2006, Ruoff *et al.* demonstrated platelets of graphite oxide with monolayer thickness (20). Now known as graphene oxide (GO), this material is electrically insulating, although it can be rendered conducting via reduction (20). The oxidation allows one to control the amount and type of attached oxides, potentially enabling control of electrical conductivity and luminescence (41). A disadvantage of this method is that it necessarily introduces chemical groups and defects that scatter electrons, giving relatively high resistivity. Graphene oxide exfoliation has been the subject of many papers and reviews (20).

Around the time of Geim's seminal paper, McEwan *et al.* produced good-quality nanographite by sonicating graphite in the solvent dichlorobenzene (42). However, monolayer graphene was not observed in this work. It was not until 4 years later that liquid-exfoliated monolayer graphene was produced by ultrasonication graphite in solvents such as *N*-methyl pyrrolidone and di-methyl formamide (27, 43). It was found that exfoliation was only achieved by using solvents with surface tension close to 40 mJ/m². Theoretical modeling showed that for such solvents, the solvent-graphene interaction is optimized so that the energetic cost of exfoliation was minimized (27, 44, 45). This method resulted in low-concentration dispersions of small but high-quality, defect-free graphene flakes. Recent improvements have substantially enhanced both the dispersed concentration (up to 30 mg/ml), the nanosheet size (up to 5 μm), and the range of solvents (28, 45–48). This method is promising for applications in which good electrical performance is required. For example, solvent-exfoliated graphene has been used to prepare transparent electrodes for liquid crystal devices (43). Recent results on printed electronic circuits have yielded mobility values of ~100 cm²/Vs (49), which are some of the highest for any chemically exfoliated graphenes. In addition, liquid exfoliation of graphene with minimal lattice defects allows the effective reinforcement of polymers (17).

Unfortunately, the most useful solvent of all, water, has a surface tension of 72 mJ/m² and so cannot by itself exfoliate graphene. However, if graphite is ultrasonicated in aqueous surfactant solutions, graphene can be exfoliated and stabilized against reaggregation through electrostatic effects (30, 50–53). Similarly, when ultrasonicated in polymer solutions, by using both water and organic solvents, graphene can be exfoliated and stabilized through steric effects

(17, 54, 55). The latter method is particularly useful as a method for producing polymer-nanosheet composites (17).

Similar methods have recently been used to exfoliate both h-BN (4, 29, 56, 57) and a range of TMDs in both solvents and surfactant or polymer solutions (29, 54, 58–61). Dispersions of TMDs such as MoS₂, WS₂, MoSe₂, MoTe₂, TaSe₂, and NbSe₂ (58) were obtained at concentrations as high as 40 mg/ml (61). As with graphene, dispersion works for solvents or solvent blends (62) with surface energies that minimize the energetic cost of exfoliation (29). TMDs prepared with this scalable method have attracted interest as composite fillers (63) and for energy storage (64). For example, Smith *et al.* have demonstrated solution-processed MoS₂/nanotube composite cathodes for Li ion batteries with reversible capacities of >200 mA h g⁻¹ (58). Chemical pretreatment also facilitates exfoliation by means of sonication in solvents. For example, functionalization allows the exfoliation of LDHs in alcohols (65). Similarly, acid treatment of metal aluminum carbides/nitrides produces the MXenes—metal carbide nanosheets with fluorinated or hydroxylated surfaces—that can be exfoliated in methanol (66, 67).

A number of advances have been made in the exfoliation of layered materials via ion intercalation. Strano *et al.* have demonstrated that dispersions of predominately bi- and tri-layer graphene can be produced from graphite intercalation compounds by thermal expansion followed by sonication in surfactant solution (21). Eda *et al.* have used ion intercalation to exfoliate MoS₂ (22), obtaining photoluminescent monolayers. Recently, Zeng *et al.* demonstrated electrochemical Li insertion followed by exfoliation for MoS₂, WS₂, TiS₂, TaS₂, ZrS₂, and graphite (68). The resultant nanosheets were fabricated into transistors and sensors. Recently, Xiao *et al.* (63) reported using exfoliated MoS₂ to prepare MoS₂/polyethylene oxide nanocomposites as battery electrodes with greatly improved lithiation capacity and cycling behavior. Du *et al.* (69) prepared MoS₂ anode electrodes by means of an exfoliation and restacking process, improving stability and achieving capacities as high as 800 mA h g⁻¹. Additionally, exfoliation by means of ion intercalation has been demonstrated for V-VI layered compounds such as Bi₂Te₃ and Bi₂Se₃ (70).

Of particular interest are layered metal oxides owing to their interesting electronic and dielectric properties (9, 10). Because of these properties, their exfoliation has been studied since at least 1990 (35) and mainly involves ion exchange followed by agitation (9). Exfoliated oxide nanosheets are most often wide-bandgap semiconductors, making them promising for applications such as photocatalysts or high-dielectric constant materials (8, 10). Alternatively, exfoliated MnO₂ and RuO₂ nanosheets are either redox-active or semi-metallic (8, 10) and have demonstrated high performance as electrochemical supercapacitors and battery materials (8, 10). Furthermore, the availa-

bility of 2D semiconducting nanosheets opens up possibilities for designing more complex nano-devices, such as photoconducting cells, p-n junctions, and field-effect transistors (FETs) (8, 9). Colloidal dispersions of exfoliated Cs₄W₁₁O₃₆²⁻ nanosheets are photochromic and superior to commonly used materials such as WO₃ (8). MnO₂ has also been exfoliated by means of ultrasonication in aqueous surfactant solutions (58), suggesting that this facile route can be applied to other layered oxides.

Intercalated layered double hydroxides were first successfully delaminated by Adachi-Pagano *et al.* by exchanging the interlayer anions with dodecyl-sulfate under reflux in butanol (71). Recent studies have focused on exfoliating such ion-exchanged materials in solvents such as formamide (71) and water (72). High-concentration (~40 g/L) dispersions of mono- and few-layer LDHs were reported by Wu *et al.* (73). Liquid exfoliated LDHs have found applications in areas as diverse as drug delivery and water treatment (8).

Although liquid exfoliation of clay minerals such as montmorillonite or vermiculites is reasonably well known, recent work has focused on dispersing clay nanosheets in hydrophobic polymers for composite applications. This has been achieved by exchanging the charge-balancing ions with organocations so as to form organoclays (74).

It has recently been shown that other layered chalcogenides such as metal phosphorous trichalcogenides (CdPS₃ and MnPS₃, for example) can also be exfoliated into single layers by means of ion exchange (75). Details of exfoliation strategies for a wide range of layered materials are given in Table 1.

Potential Applications of Liquid-Exfoliated Nanosheets

Liquid exfoliation represents a versatile, scalable, and sustainable route for production of 2D nanosheets. In addition, access to suspensions of nanosheets permits processing in ways that would otherwise be difficult or impossible. For example, processing from liquids allows the deposition of individual nanosheets on surfaces and the formation of thin or free-standing films; facilitates mixing with other nanomaterials to form hetero-structured solids; and enables insertion, as fillers, into polymer matrices (Fig. 4).

The ability to invoke different phases of 2D materials via tuning the exfoliation chemistry opens up an exciting range of possibilities. For example, exfoliation via electrochemical ion intercalation leads to realization of the metallic (1T) phase in layered transition metal chalcogenides, which cannot be accessed through other synthesis techniques (76). Some applications include electrodes for energy-storage devices, high-performance fillers for polymer-based composites, electro- and photocatalysis for hydrogen evolution, nano- and large-area electronics and opto-electronics, and low-friction additives in lubrication systems.

The production of inexpensive 2D nanosheets holds promise for multifunctional polymer com-

posites. Polymer-clay composites can display improved mechanical and barrier properties, usually at loading levels from a few percent to a few tens of percent (77). Because of the potentially high aspect ratio of exfoliated graphene, polymers loaded with graphene display reinforcement (17), conductivity enhancement (78), and barrier properties (79) at loading fractions below 1 volume %. Solvent processing of 2D nanosheets makes composite fabrication straightforward and will extend the suite of 2D fillers beyond clays and graphene (4, 29, 80). Liquid exfoliation also allows the formation of unusual composites consisting of mixtures of nanosheets, nanotubes, and other nanostructures (Fig. 4) (29, 58). This results in synergetic effects; for example, composites of WS₂ and carbon nanotubes display very high conductivity but retain much of the character of WS₂ films (29, 58, 81). Such composites are promising for applications in photovoltaic or thermoelectric devices and as electrodes for supercapacitors or batteries.

Supercapacitor electrodes formed from chemically exfoliated graphene nanosheets have yielded energy densities of 20 W h kg⁻¹ for packaged devices, approaching those of lead-acid batteries (82). Recent results on restacked nanosheet films indicate that they maintain their structural integrity upon intercalation and deintercalation (58). The wide range of 2D materials available with varying electrochemical properties makes them ideal for energy storage. Initial measurements indicate that their capacitive capability is very high, although issues relating to substantial loss after the first few cycles remain. Choosing the correct combinations of materials will allow multiple properties (such as surface area, conductivity, and electrochemical stability) to be simultaneously improved so as to provide flexibility for designing electrodes for batteries and supercapacitors. For example, materials such as VS₂, MoO₃, and MnO₂ could be useful for efficient energy storage.

The possibility that layered materials could catalyze the production of hydrogen from water, for use as a fuel, is a critically important application. The edge molybdenum sites on TMDs are highly active for hydrogen evolution reactions and thus are being considered as electrodes for water-splitting by using sunlight. With ion-exfoliated 2D TMDs, the presence of a metallic (1T) phase makes the entire basal plane catalytically active, leading to an improvement in the catalytic activity and a substantial increase in the number of active catalytic sites (83). Oxide nanosheets (such as TiO₂ and MoO₃) are also candidates for hydrogen evolution catalysts (84).

Liquid-phase exfoliation facilitates the integration of the materials into large-area electronics through the well-known solution-based techniques such as inkjet printing (49) and roll-to-roll coating. Recent results have indicated that the electronic structure of the 2D nanosheets is largely preserved after liquid exfoliation so that fundamental processes can be observed in individual

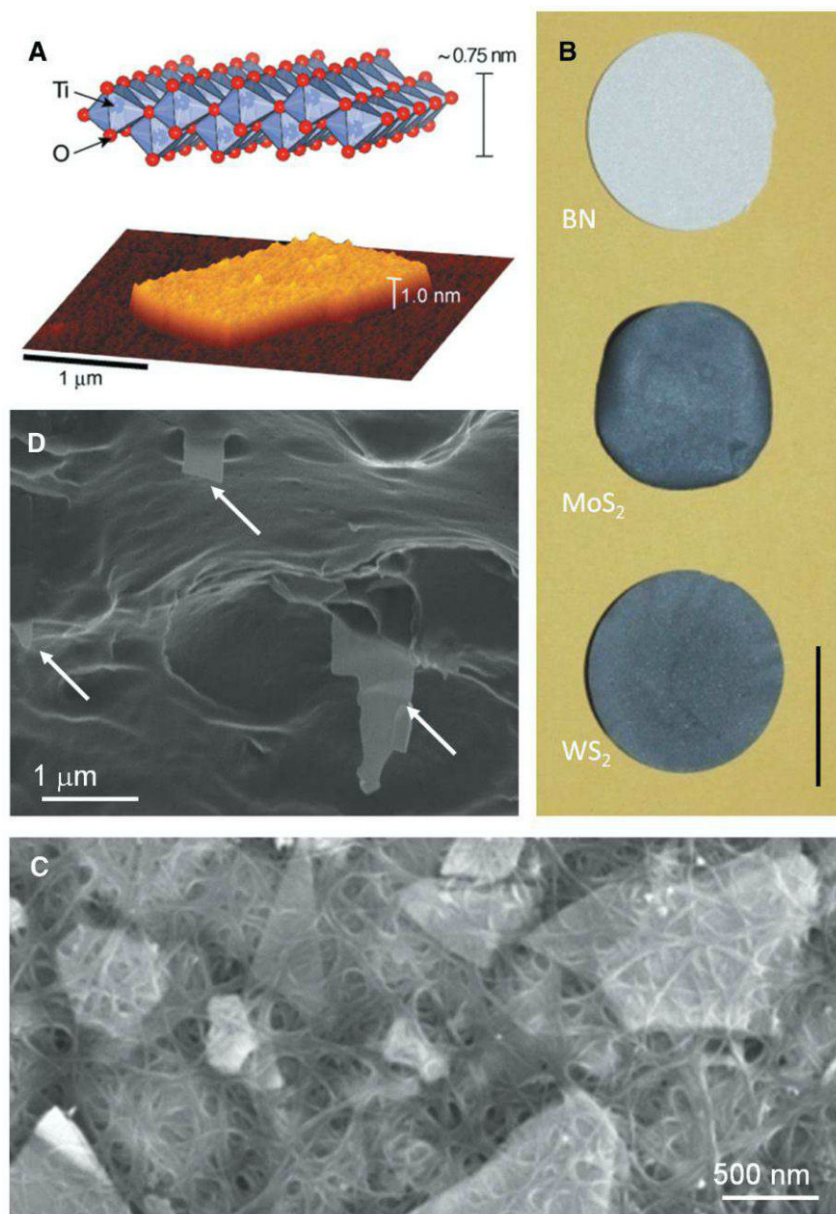


Fig. 4. Once layered materials have been exfoliated in liquids, they can be easily processed into a range of structures. (A) TiO₂ nanosheets deposited onto a substrate and imaged by means of AFM (10). **(B)** Solution-processed, free-standing films of randomly arranged nanosheets of h-BN, MoS₂, and WS₂ (29). Scale bar, 25 mm. **(C)** A composite film of WS₂ nanosheets in a matrix of randomly arranged carbon nanotubes (29). **(D)** Solvent-exfoliated graphene nanosheets (arrows) embedded in a polymer matrix (17). All images were adapted from the sources as referenced.

sheets. For example, because exfoliated oxides retain their excellent dielectric properties, they can be used as high- κ dielectrics in nanoelectronics (10). Alternatively, stacking of different layered perovskites allows the fabrication of artificial multi-ferroic materials for use in future oxide-based electronics and memory devices. Exfoliated oxide nanosheets may also be used in more complex nanodevices, such as photoconducting cells, p-n junctions, and field-effect transistors (8, 10). Alternatively, MoS₂ shows exceptionally stable transistor operation under very large mechanical deformation (85). The relatively undisturbed electronic structure coupled

with flexibility makes MoS₂ and other 2D materials useful for next-generation large-area electronics and opto-electronics on plastic and paper platforms. In particular, large-area, solution-cast, TMD thin-film transistors with mobility of ~ 10 to $50 \text{ cm}^2/\text{V s}$ could replace doped oxide semiconductors for back-plane switching in flexible and transparent displays.

More exotic applications of layered 2D materials will arise from the stacking and seamless integration of specific combinations of materials in order to access fundamental properties such as superconductivity and condensates (86). The integration could be in the vertical or lateral direc-

tion so as to allow for different device concepts. The ability to access metallic and semiconducting phases in 2D materials with electrochemical exfoliation also provides opportunities for designing molecular-scale heterostructures with atomically coherent junctions (24). These types of superlattice structures could be easily synthesized from liquid dispersions.

Outlook

Our ability to employ straightforward techniques to exfoliate materials points to a productive future in this area. However, a number of substantial challenges remain. Although ultrasonication-assisted solvent exfoliation displays great promise, it also has a number of limitations in its current form. For example, the monolayer yield is generally no more than a few tens of percent by number, which is far too low for many applications. As such, it will be necessary to develop methods to monitor and increase both the monomer yield and indeed the lateral flake size. For ion intercalation-based methods, the time required to achieve exfoliation must be reduced. Crucially, the scale-up of liquid exfoliation must be demonstrated. The ultimate aim must be production on an industrial scale (more than kilograms per day)—a goal that seems relatively far off for defect-free materials. This will almost certainly require exfoliation methods that do not rely on ultrasonication, a method that has limited scalability. However, an achievable early target would be a production rate of grams of exfoliated nanosheets per hour.

It will be important to demonstrate exfoliation of a wider range of layered materials. To date, liquid exfoliation has produced graphene, h-BN, a number of TMDs, a range of clays, many oxides and hydroxides, and a scattering of other exfoliated nanosheets. However, there is a galaxy of other materials (Table 1) offering a rich set of distinct features that have not yet been exfoliated but should be amenable to the methods described above. For example, because layered GaSe and InSe are photoluminescent, liquid exfoliation could lead to next-generation solution-processed, photostable light-emitting diodes. Alternatively, V-VI layered compounds (such as Bi₂Te₃ and Bi₂Se₃) could form the basis of solution-processed thermoelectric materials (29, 58).

In addition, exfoliation in liquids will be a good starting point for subsequent chemical modification of nanosheets. For example, chemical functionalization will allow controlled modification of the properties of exfoliated nanosheets for applications in composites, targeted drug delivery, or sensing. It will be critical to develop the chemistry required to attach a wide range of chemical groups to both nanosheet basal plane and edge in a controlled way.

Last, there are a number of cases in which 2D materials might be produced from starting materials that differ considerably from the layered crystals described above. Specifically, we consider materials that lack a clearly defined van der

Waals gap. There are entire families of materials that are made of extended layers carrying negative or positive charges and are separated by charge-balancing cations or anions, respectively. Such systems will have just as interesting chemical and physical properties as the neutral layered analogs. Some examples include the semiconducting NaInSe_2 , $\text{K}_2\text{Cd}_3\text{S}_4$, $\text{K}_2\text{Fe}_4\text{Se}_5$, the metallic KCu_4S_3 , BaFe_2As_2 , and the superconducting KFe_2As_2 . Instead of weak van der Waals forces, much stronger electrostatic interactions hold these layers together. In these cases, separation of the layers will require the apportioning of the charge-balancing ions between them to partition evenly on both layers coming apart. This will create surfaces that need to be neutral. Therefore, innovative chemical methods that can dismantle the solid-state lattices and stabilize such surfaces will be required. That such approaches are feasible is evidenced by the chemical modification and subsequent exfoliation of Ti_3AlC_2 —a material without a van der Waals gap—to give nanosheets of $\text{Ti}_3\text{C}_2\text{F}_2$ and $\text{Ti}_3\text{C}_2(\text{OH})_2$ (67).

As exfoliation methods increase in their sophistication and effectiveness, layered materials will yield a host of new 2D systems to explore. The diverse range of properties of known layered crystals means that their exfoliated counterparts will be equally diverse, leading to applications in electronics, photonics, energy storage, structural composites, drug delivery, barrier layers, and coatings, to name but a few. We envisage exfoliated nanosheets becoming central to nanotechnology in the 21st century.

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Pliocene Warmth, Polar Amplification, and Stepped Pleistocene Cooling Recorded in NE Arctic Russia

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Understanding the evolution of Arctic polar climate from the protracted warmth of the middle Pliocene into the earliest glacial cycles in the Northern Hemisphere has been hindered by the lack of continuous, highly resolved Arctic time series. Evidence from Lake El'gygytyn, in northeast (NE) Arctic Russia, shows that 3.6 to 3.4 million years ago, summer temperatures were ~8°C warmer than today, when the partial pressure of CO₂ was ~400 parts per million. Multiproxy evidence suggests extreme warmth and polar amplification during the middle Pliocene, sudden stepped cooling events during the Pliocene-Pleistocene transition, and warmer than present Arctic summers until ~2.2 million years ago, after the onset of Northern Hemispheric glaciation. Our data are consistent with sea-level records and other proxies indicating that Arctic cooling was insufficient to support large-scale ice sheets until the early Pleistocene.

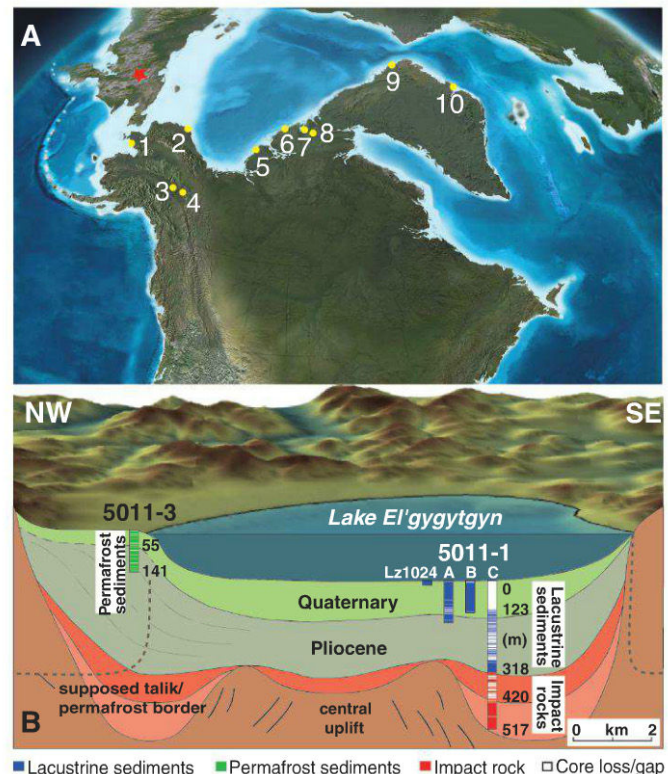
The geologic record makes it clear that global temperatures are intimately linked to changes in atmospheric greenhouse gas concentrations (1). Today's CO₂ levels are similar to Pliocene estimates [4.5 to 2.588 million years ago (Ma)], ranging from 380 to 450 parts per million (ppm), or some 30% higher than preindustrial levels (2, 3). Pliocene warmth, considered an analog for future climate scenarios, persisted across repeated warm and cool Milankovitch cycles, raising questions concerning climate sensitivity to CO₂. Moreover, it remains unclear what mechanisms drove the amplification of polar temperatures and past reductions in sea ice (4, 5) and the eventual cooling and growth of continental Northern Hemispheric ice sheets. The PRISM climate mapping program (6) compiled important global climate information for the interval from 3.26 to 3.02 Ma but overlooked

the earlier middle Pliocene, millennial-scale variability of cooler Pliocene climate states, and the Pliocene/Pleistocene transition [boundary at 2.588 Ma (7)]. The terrestrial Arctic is rich in Pliocene stratigraphic sequences that docu-

ment time slices when conditions were much warmer than today (8–10). Yet marine sediments on the Lomonosov Ridge suggest that the Arctic Ocean has maintained perennial sea ice (11–13) or perhaps periods of seasonal sea ice since the middle Miocene (14). The discordance between terrestrial and Arctic marine records has remained unresolved largely because of the lack of continuous land records for comparison (15, 16).

Here we present results from a continuous, middle Pliocene to early Pleistocene lacustrine record from the terrestrial Arctic, derived from Lake El'gygytyn, located 100 km north of the Arctic Circle in Chukotka, northeastern Russia (67°30'N, 172°05'E; Fig. 1). The basin was created by a meteorite impact 3.58 ± 0.04 Ma (17) and contains a continuous sediment sequence recording a complete Late Cenozoic climate history on centennial to millennial time scales. The data here demonstrate extreme warmth and polar amplification during the middle Pliocene, stepped cooling events during the Pliocene-Pleistocene transition, and the recurrence of Arctic summer warmth until after 2.2 Ma, after the paced onset of Northern Hemispheric glaciation (3, 18, 19). Recurring but declining warmth in the Arctic borderlands has implications for understanding the transition from a forested Arctic in the middle Pliocene to the tundra-dominated landscape of today. Moreover, the data raise questions concerning

Fig. 1. Location of Lake El'gygytyn and stratigraphic cross section. (A) Lake El'gygytyn (star) formed 3.58 Ma in NE Russia, when North America remained a contiguous landmass (and had a more continental climate), unbroken by tectonics and glacially eroded marine troughs (33) (map courtesy of Ron Blakely). Selected Pliocene and early Pleistocene sites mentioned in the text and supplemental materials are shown (1, Nome; 2, Gubik Formation; 3, Klondike Area; 4, Lost Chicken; 5, Banks Island; 6, Hvitland Beds; 7, Meighen Island; 8, Strathcona Fjord; 9, Kap København; 10, Lodin Elv). (B) Schematic cross-section of the El'gygytyn basin stratigraphy, showing the location of ICDP sites 5011-1 and 5011-3. At site 5011-1, three holes (1A, 1B, and 1C) were drilled to replicate the Quaternary and uppermost Pliocene sections. Hole 1C penetrated the remaining lacustrine sequence down to a depth of 318 m and then ~200 m into the impact rock sequence underneath. Lz1024 is a 16-m-long percussion piston core taken in 2003 that fills the stratigraphic gap between the lake sediment surface and the top of drill cores 1A and 1B (18).



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the seasonal persistence of Arctic sea ice in the past, the evolution of Northern Hemisphere ice sheets, and the onset of major glacial-interglacial cycles (20, 21).

Lake Setting, Core Acquisition, and Methods

At the time of the El'gygytyn impact 3.6 Ma (17), the North American Arctic had a more continental geography (Fig. 1), forests reached the Arctic Ocean coast (10), Greenland was mostly ice-free (22, 23), and permafrost was not widespread (10, 24). Today Lake El'gygytyn is 170 m deep and 12 km in diameter, with an area of 110 km² within a catchment of only 293 km² (25). It is cold, monomictic, and oligotrophic, with a summer temperature of ~3°C. Distinct lake-level changes are recorded by terraces and wave-cut notches as high as 35 to 40 m above and 10 m below the modern lake level, with the highest benches occurring in the early basin history (26, 27).

The Lake El'gygytyn region today has a mean annual air temperature (MAT) of ~-10°C, with mean July temperatures of ~+8°C and average winter lows of ~-35°C. Mean annual precipitation (PANN) is about 200 mm/year. Reanalysis data show that the climatology of the basin is representative of much of the western Arctic (25). Although the lake area currently lies within the subzone of southern shrub tundra, today's climatology supports only sparse vegetation, dominated by hummock and moss tundra over deep permafrost with some prostrate willows and dwarf birch.

Drilling at Lake El'gygytyn occurred in spring 2009 from a lake-ice platform (28). Lake basin stratigraphy is based on seismic surveys (29) suggesting continuous sedimentation over the time period of interest. The 318-m-long composite sequence representing the complete 3.6 Ma record in International Continental Scientific Drilling Program (ICDP) core 5011-1 (Fig. 1) was investigated using initial nondestructive scanning and logging technologies along with multiproxy investigations on subsamples (supplementary materials). The core chronology is based on magnetostratigraphy and tuning of proxy data with marine isotope stratigraphy (21) and summer insolation at 67.5°N (30) (supplementary materials and figs. S4 and S5).

The sedimentology is composed of five pelagic facies (Fig. 2D; supplementary materials and figs. S1 and S2). One striking feature of the sediments is the remarkably cyclic nature of many proxies, including biogenic silica content (BSi%) and accumulation rate (BSi acc. rate), Si/Ti ratio, and the concentration of total organic carbon (TOC) throughout the record younger than 2.65 Ma (18) (Fig. 2, E and F, and fig. S4). BSi percentage and acc. rate and Si/Ti ratios are shown to be robust proxies of the lake's primary productivity, with higher values generally associated with high summer insolation and relatively light marine isotopic values (Fig. 2, A and B).

Similar regular cycles are not seen >2.65 Ma. In contrast, high productivity is implied by high Si/Ti ratios and BSi acc. rate from 3.3 to 3.1 Ma (Fig. 2, E and F), but in the early lake history before 3.2 Ma, the productivity signal is diluted by higher clastic flux rates, as evoked by the BSi acc. rate, low Si/Ti ratios, and higher sedimentation rate (Fig. 2E and fig. S3).

Mid-Pliocene Extreme

Our Lake El'gygytyn record provides a continuous terrestrial record showing the extreme Arctic warmth from 3.6 to 3.4 Ma that other, shorter records of the middle Pliocene have inferred (31). The earliest sediment history in the lake begins a few thousand years after the impact, as hydrothermal activity ceased and the crater cooled and filled with water. The first 17 m of post-impact sediment (~the first 20,000 years) is almost devoid of pollen and other microfossils, but after 3.56 Ma, the pollen stratigraphy provides the best descriptor of paleoenvironmental conditions (supplementary materials). The mean temperature of the warmest month (MTWM, Fig. 2H) and PANN (Fig. 2I) reconstructed from the pollen record imply values of +15° to 16°C and ~600 mm/year, respectively. These estimates are 7° to 8°C warmer and 400 mm wetter than today. Similar values were reconstructed for the Pleistocene super-interglacial marine isotope stage (MIS) 11c (18), but this time interval lacks fir and hemlock pollen, highlighting the exceptional warmth and wetter conditions of the middle Pliocene. Cooler intervals of the record occur at 3.46 and 3.42 Ma during summer insolation minima and maxima in the marine oxygen isotope stack (e.g., MG6).

Elevated BSi deposition (Fig. 2E) suggests greater seasonal productivity and is consistent with generally warmer and wetter conditions between 3.56 and 3.4 Ma. The dominant planktonic diatom (*Pliocaenicus* sp.) is significantly larger there than during any other interval in the entire lake record, suggesting extremes in nutrient delivery and reduced seasonal ice cover. This is consistent with higher rates of siliciclastic sedimentation, probably caused by increased precipitation, steeper relief, and reduced or absent permafrost within the catchment (supplementary materials, fig. S3).

Mid-Pliocene warmth has previously been documented in other parts of the terrestrial Arctic, but those records are limited in duration, precluding comparisons with orbital forcing. Nonetheless, they provide a vital spatial network of sites for characterizing the Arctic borderlands. The Strathcona Fjord Beaver Pond dated to 3.4 Ma (32) (Fig. 1, site 7) spans ~40,000 years. Reconstructed MATs were continuously +19°C warmer than today (-1.6°C), and summer growing season temperatures were in the range of 14°C (8, 9, 32). The Canadian Archipelago remained a contiguous landmass at this time (33) (Fig. 1), with forests likely extending to the coast of the Arctic Ocean (10). At the same time, sea

surface temperatures (SSTs) in the North Atlantic were 18°C [-7°C warmer than today (Fig. 3D) (34)], and latitudinal temperature gradients were lower (35, 36) (Fig. 3H). In Antarctica, persistent open-water conditions in the Ross Embayment and a reduced West Antarctic Ice Sheet from 4.5 to 3.4 Ma imply 1.2 million years of continuous warmth (37) (Fig. 3J). Hence, in the mid-Pliocene, both polar regions were substantially warmer than at present. At Lake El'gygytyn, warm conditions continued even during orbits with reduced boreal summer insolation (Fig. 2, B versus H).

Pliocene-Pleistocene Warmth

Reconstructed MTWM (Fig. 2H) and PANN (Fig. 2I) from 3.26 to 2.2 Ma also indicate a protracted period of warm and moist conditions ~3° to 6°C warmer and <100 to >200 mm wetter than in modern times and warmer than the last interglacial MIS 5e (18). Beyond the exceptional vegetation response (Fig. 2, G to I), BSi (Fig. 2E) and TOC (fig. S4) are also elevated during most of this time. Important exceptions to these warm conditions include marine isotopic intervals labeled MIS 100, 96, and 86 (Fig. 2A), when MTWM estimates dip near or below modern values (Fig. 2H) and approach cold temperatures similar to those estimated by the same proxies for late Pleistocene glacial conditions [the Last Glacial Maximum (LGM), Fig. 3A]. Summers cooler than today are largely younger than 2.5 Ma, with only one exception, the M2 cooling event at ~3.3 Ma. Even most early Pleistocene temperatures remained higher than those of the middle and late Pleistocene.

Changes in boreal forest composition occur with the loss of conifers at 2.71 to 2.69 Ma and then the transition from forested habitats to treeless and shrubby environments in steps at 2.6, 2.56, and 2.53 Ma (Fig. 3, G and I). These changes provide estimates of the mean temperature of the coldest month (MTCM; fig. S8), with important implications for Beringian vegetation-climate feedbacks (38) as expanding tundra increased land surface albedo, especially during snow-covered months. These cooler vegetation types become dominant <2.53 Ma, even during high summer insolation orbits, except during super-interglacials 93, 91, and 87 (18) (supplementary materials). Climate-vegetation model simulations using 300 and 400 ppm CO₂ (Fig. 4 and supplementary materials) are consistent with forest cover at Lake El'gygytyn before ~2.53 Ma and restricted glacial ice over Greenland in both cold and warm boreal summer orbits (39), especially for the PRISM interval from 3.26 to 3.02 Ma (6).

Cross-correlation of MTWM (Fig. 3A) with the LR04 stack (Fig. 3B) provides a rather good fit [correlation coefficient (R^2) = 0.41, fig. S8], illustrating the expected link between summer temperatures at Lake El'gygytyn and obliquity via the isotopic stack (Fig. 3C). Several intervals

of coniferous-dominated forests occurred during MIS 101, 93, 91, and 87 interglacials (40). Summers at Lake El'gygytyn remain 1° to 3°C warmer than at present for ~200,000 years after

stratification of the North Pacific at 2.73 Ma (41) (Fig. 3F), even during the coldest boreal summer orbits (MIS G6, Fig. 2H). These data, along with eustatic sea-level reconstructions (Fig. 3E),

support the notion that Pleistocene cooling and the intensification of Northern Hemisphere glaciation (NHG) were gradual, occurring in several steps (5, 34–36), but with warm Arctic

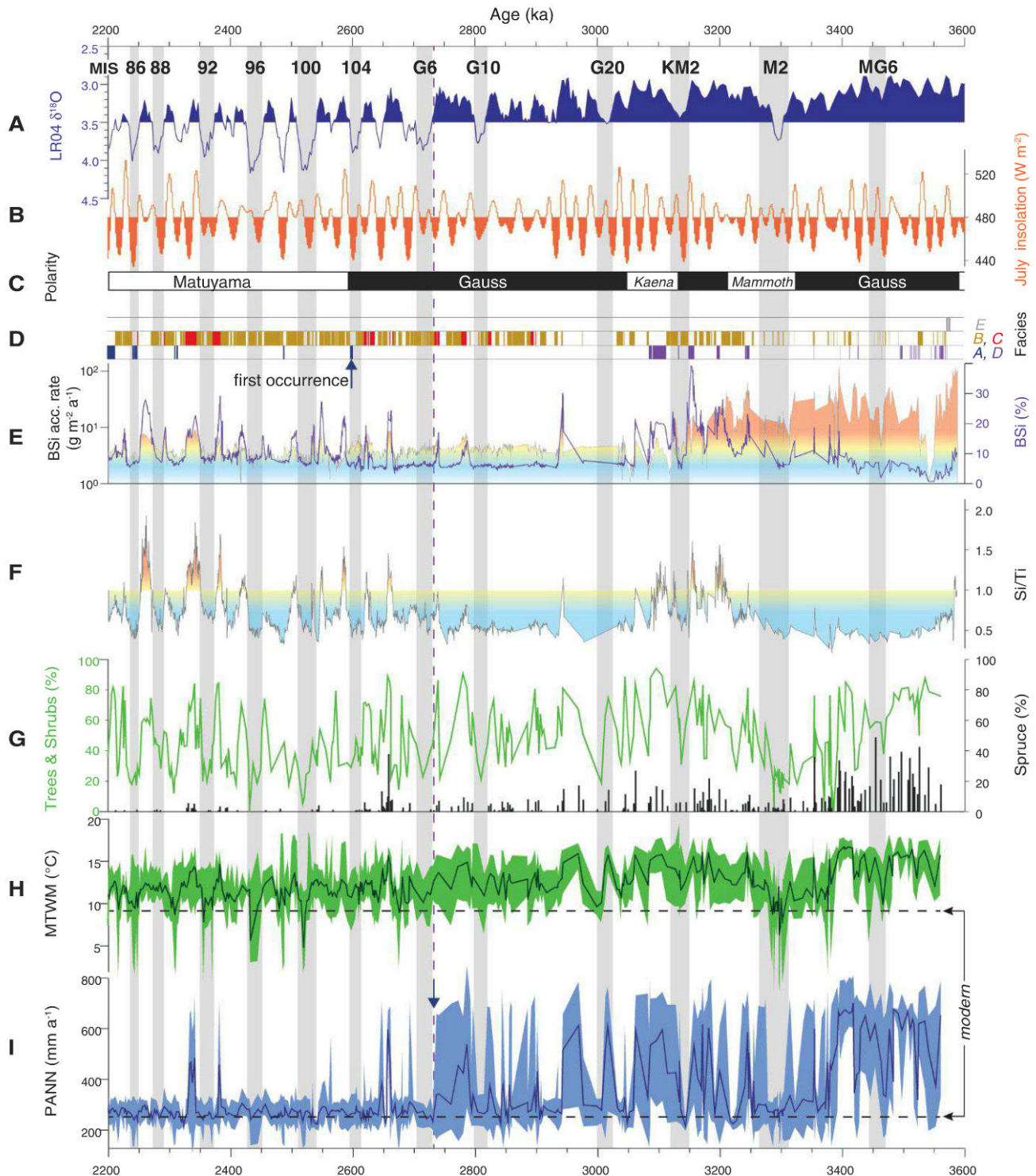


Fig. 2. Chronology, sediment facies, and proxy data from 3.58 to 2.2 Ma from Lake El'gygytyn. (A) Global marine isotope stack (21) (ka, thousand years ago) and (B) mean July insolation for 67.5°N (30), next to (C) the magnetostratigraphy, (D) sediment facies defined in the text and supplementary materials, (E) FTIRS-inferred biogenic silica plotted as BSi acc. rate (note logarithmic scale) and % BSi, and (F) Si/Ti ratio. These

data are compared to (G) percent of trees and shrubs (green line) and spruce (black bars) pollen. (H) Reconstructed MTWM and (I) annual precipitation (PANN), based on the pollen spectra and the best modern analog approach applied in (18). The darker colored line in both (H) and (I) represents the mean values, whereas colored shading is the uncertainty range [modern values from (63)].

summers persisting much later than previously documented.

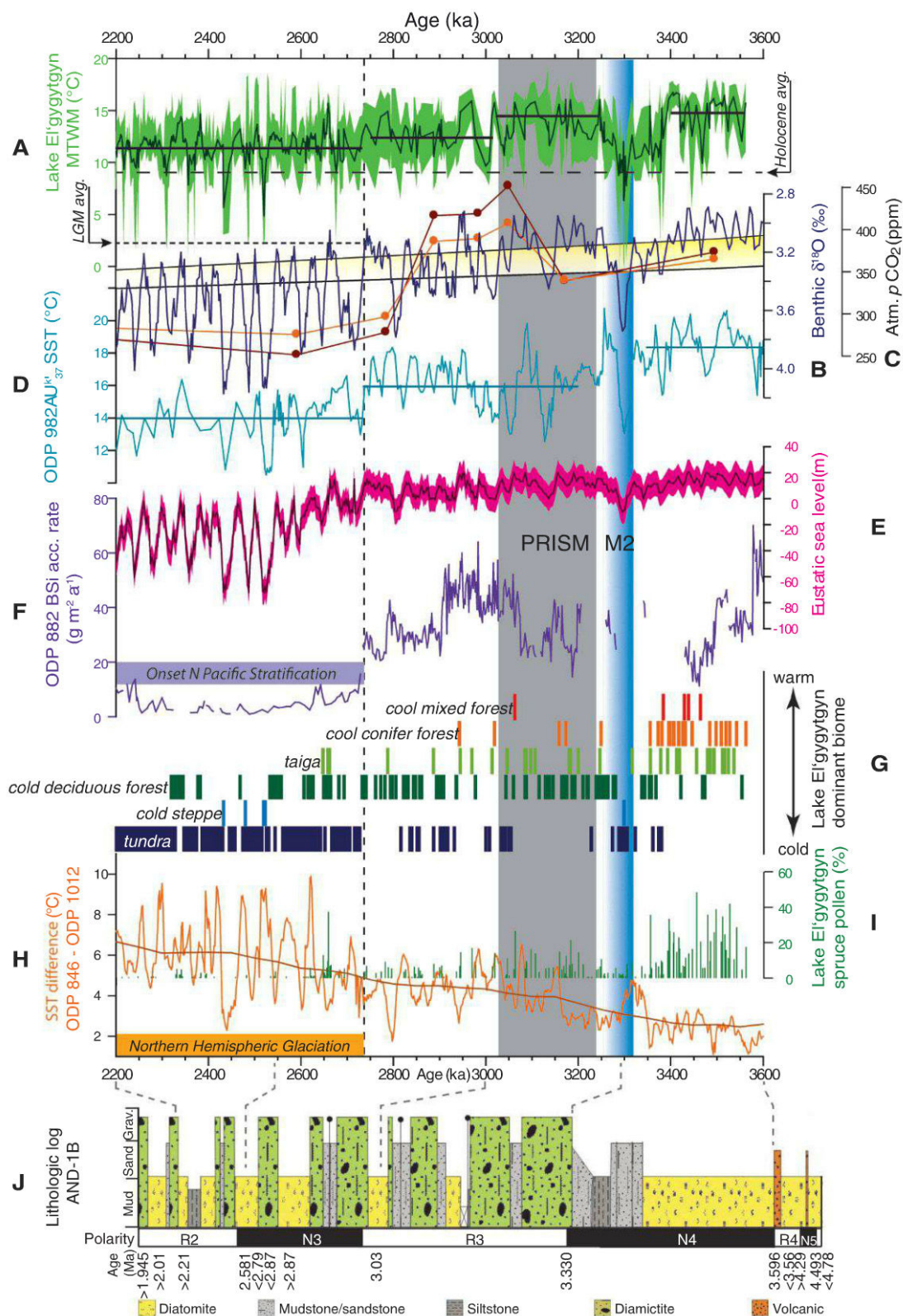
At Lake El'gygytyn, the PRISM interval (Fig. 3) was nearly as warm as it was between 3.56 and 3.4 Ma, and boreal forests changed little in composition (Fig. 3G). At Meighen Is-

land (Fig. 1, site 6), fossil plant and beetle assemblages suggest July temperatures $+10^{\circ}$ to 11°C warmer than today (42). Data from several sites show that boreal forests stretching from 60° to 80°N included pine, not found in modern forests of Alaska or the Yukon (31).

Yet the average MTWM reconstructed over the PRISM interval is slightly warmer ($\sim 1.5^{\circ}\text{C}$) than in multimodel climate simulations of the Pliocene Model Intercomparison Project (PlioMIP) (43). In the PlioMIP simulations using eight different coupled atmosphere-ocean general circula-

Fig. 3. Global proxies of change compared to Lake El'gygytyn.

(A) MTWM from Fig. 2, with solid horizontal lines shown as mean values over the selected intervals; the long dashed line is the Holocene average temperature, and the short dashed line is the LGM average temperature from (18). (B) Benthic marine LR04 stack (21); (C) changes in $p\text{CO}_2$ shown as a range window in yellow, estimated from minimum and maximum of (2), with low-resolution estimates using boron methods (orange and red solid circles) in (3); (D) alkenone reconstructed SSTs for the North Atlantic (34) (ODP, Ocean Drilling Program); (E) eustatic sea-level record from New Zealand, representative of the global stack (55); (F) mass accumulation rate of biogenic silica from the North Pacific (41); (G) dominant reconstructed biomes from Lake El'gygytyn; (H) change in SST gradient between the North Pacific tropics and subtropics from (59); (I) percent of spruce pollen; compared with (J) lithology of ANDRILL core ANT-1B from a depth of 438 to 190 m in (37). The blue vertical shading is the M2 isotopic event, and the gray vertical bar is the PRISM research interval. The dashed vertical line marks 2.72 Ma.



tion models (43), the multimodel MAT at Lake El'gygytyn is $+7.05 \pm 2.7^\circ\text{C}$ warmer than in preindustrial times and the MTWM is $5.5^\circ \pm 2^\circ\text{C}$ warmer. The simulated MTWM is close to the PRISM-average reconstructed warming of $\sim 7^\circ\text{C}$. However, at times during PRISM, MTWM temperatures at the lake were $>8^\circ\text{C}$ (Fig. 3A) warmer than present. In sum, model sensitivity to Pliocene boundary conditions, including atmospheric CO_2 , appears to be slightly lower than the observed sensitivity at Lake El'gygytyn.

M2, Pliocene Transition, and Pace of Pleistocene Cooling

The pollen-based reconstructions from Lake El'gygytyn show that the largest cooling event of the mid-Pliocene took place from 3.31 to 3.28 Ma (MIS M2) (21). Conditions were cooler and dryer, as documented by a dramatic decrease in the relative abundance of arboreal pollen and increases in *Artemisia*, Poaceae, and other herbaceous pollen (fig. S6). Although larch, pine, birch, alder, and possibly spruce stands remained around the lake, steppelike habitats dominated

(Fig. 3G). Coprophilous fungi indirectly point to the presence of large grazing animals, such as mammoth, horse, and bison (40). Climatic conditions were not "glacial"; rather, it was as warm as the Holocene average or slightly warmer (44) (Fig. 3A). This has implications for interpreting the M2 isotopic shift of ~ 0.5 per mil in the North Atlantic (21). Modeling sensitivity studies (39) do not preclude the existence of a Greenland Ice Sheet during M2 as a major source of ice-rafted debris (IRD) (45). However, if Holocene-like conditions at Lake El'gygytyn are indicative of broader circum-Arctic climate, conditions for the initiation of ice over North America would have been unfavorable until long after 3 Ma (46). Our results are consistent with gradual alkenone-based evidence of SST cooling in the eastern North Atlantic in the range of 2° to 3°C (47) and 6°C (34) during M2, but with ocean temperatures remaining warmer than modern ones both before and after (Fig. 3D). Our findings are also consistent with drilling results from the Ross Sea (ANDRILL), suggesting that most M2 ice advance outside Alaska, Greenland, and Svalbard

took place in Antarctica rather than North America (Fig. 3J) (4, 37, 48).

The gradual but paced onset of NHG took place as step changes starting after 3.6 Ma (49), but temperatures in the Arctic remained elevated until ~ 2.2 Ma. Although records of IRD from the North Atlantic/Barents Sea (50) and the North Pacific (41) indicate the presence of glacial margins reaching tidewater, IRD records do not provide a measure of ice-sheet size. Eustatic-equivalent changes in sea level, in contrast, offer an index of global ice volume (21) but not where the ice was located (51).

Direct evidence of North American continental-scale ice sheets extending southward from Canada does not appear until after 2.4 Ma (52, 53) and may have had a different geography with warm-based, subglacial conditions (54). This may explain why sea-level variability during initial cold cycles after 2.6 Ma (MIS 100) was in the range of ~ 70 m, which is much less than ~ 125 m for the LGM (Fig. 3E) (55). In the Alaskan Brooks Range, the earliest glacial cycles were after 2.7 Ma and were most extensive toward the Arctic Ocean,

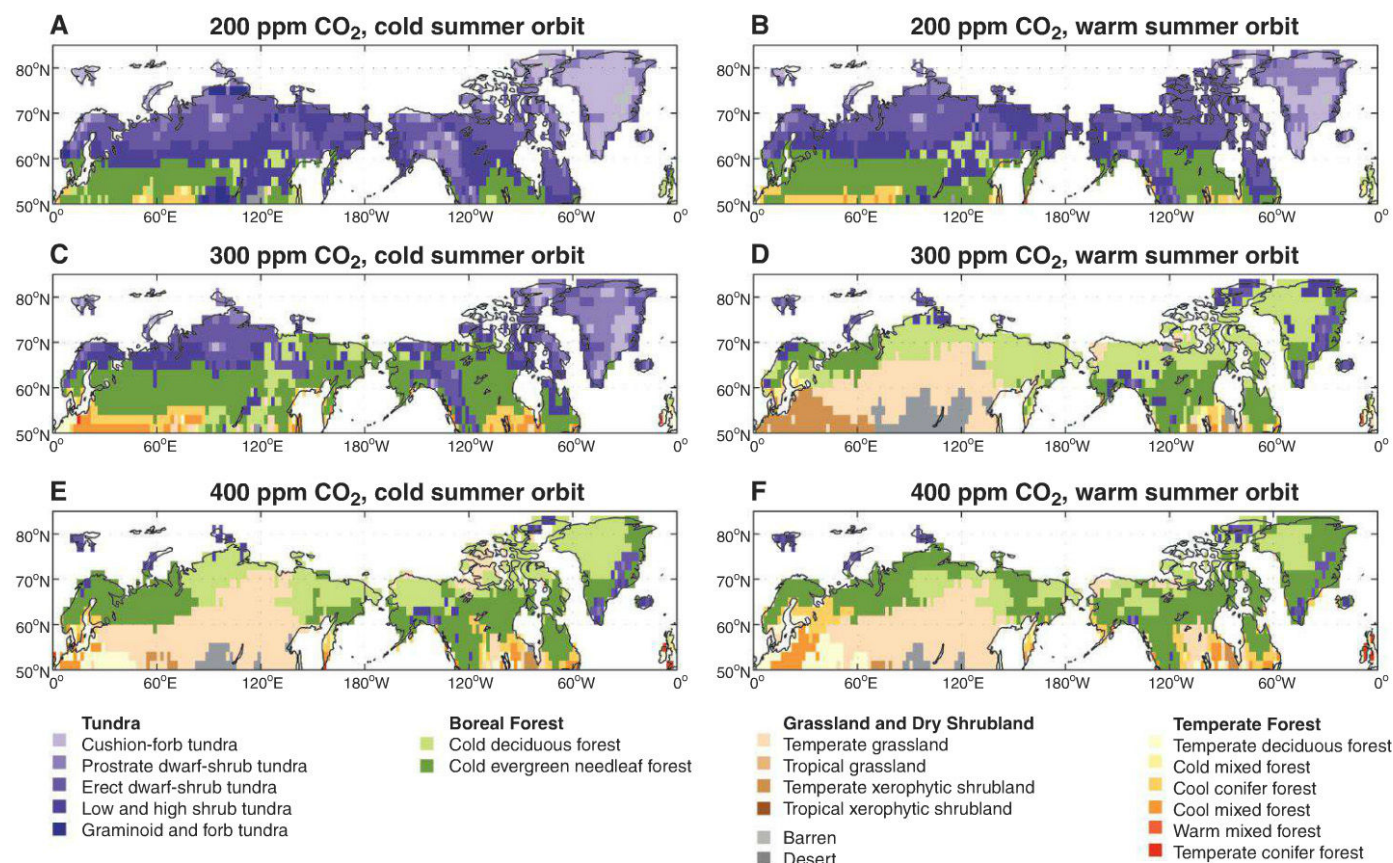


Fig. 4. Coupled climate-vegetation model simulations spanning the Pliocene-Pleistocene transition. All simulations assume an ice-free Northern Hemisphere, including Greenland. CO_2 atmospheric mixing ratios span the range assumed to have existed at the onset of major Pleistocene glacials [A and B; 200 ppm by volume (ppmv)], during the Pliocene-Pleistocene transition (C and D; 300 ppmv), and in the warm Pliocene (E and F; 400 ppmv). (A), (C), and (E) assume an orbital configuration like that at 116 ka, producing relatively cold boreal summers, whereas (B), (D), and (F) use a warm boreal summer orbit like

that at 125 ka. The simulations place the pollen-based vegetation reconstruction (Fig. 3G) into a spatial and temporal context, relative to proxy-based CO_2 reconstructions. Although the persistent dominance of forest biomes at Lake El'gygytyn until 2.6 Ma is in broad agreement with the model, the lack of temperate forest at 400 ppm of CO_2 demonstrates either the undersensitivity of the model to CO_2 forcing or the possibility that Pliocene greenhouse gas levels were higher than in most proxy reconstructions. Descriptions of the model and boundary conditions are provided in the supplementary materials.

suggesting that a more sea-ice-free Arctic Ocean may have provided an important precipitation source (56). A major drop in the partial pressure of CO_2 ($p\text{CO}_2$) at ~2.8 Ma that could have driven additional cooling is indicated in some proxy records (3) (Fig. 3C). Increases in ocean stratification and IRD in the North Pacific from the mountainous Pacific Rim at 2.73 Ma (57), when inferred $p\text{CO}_2$ is in the range of 280 to 370 ppm (2, 3) (Fig. 3C), coincide with a significant drop in precipitation at Lake El'gygytyn (Fig. 2I) and an abrupt change in boreal forest composition, characterized by vegetation still requiring summer temperatures warmer than the Holocene average (Fig. 3A). Thus, our vegetation record is consistent with estimates for $p\text{CO}_2$ (2, 3), but Arctic temperatures consistent with that vegetation make it difficult to reconcile the buildup of major continental ice sheets with summers warmer than today, even during cold Arctic summer orbits. The substantial PANN drop at 2.73 Ma (Fig. 2I) and onset of cold winter temperatures, at times colder than today (fig. S8), are consistent with preliminary model simulations showing the climatic impact of large Northern Hemispheric ice sheets on the Arctic basin and Beringia (fig. S9).

At Lake El'gygytyn, the first cold "glacial" sediment, Facies A (Fig. 2D), occurs at ~2.6 Ma (18); this facies, indicative of perennial summer lake-ice cover and MAT at least $4^\circ \pm 0.5^\circ\text{C}$ colder than today (58), becomes common after 2.3 Ma during cooler summer orbits, after an increase in low latitudinal temperature gradients in the Pacific (Fig. 3H) (59). Perennial lake-ice cover implied by Facies A does not appear during all cold orbits until after 1.8 Ma (18). Likewise, the first cold interval in Lake Baikal occurs at 2.7 Ma, but cyclic patterns of glacial/interglacial BSi do not take hold until after about 1.8 Ma (36, 60).

Warm temperatures and changing boreal forest composition at Lake El'gygytyn, together with other proxies, contribute to a growing body of marine and terrestrial evidence that climate system change from the warm Pliocene to the late Pleistocene glacial world was a punctuated sequence of stepped Northern Hemispheric cooling events and ice buildup (34, 36, 49, 59). Although latitudinal temperature gradients steepened after 2.65 Ma (Fig. 3H), swings in global sea level increase only after 2.6 Ma (Fig. 3E), despite warmer Arctic summers, which is consistent with the waxing and waning of the West Antarctic Ice Sheet (Fig. 3J) (37). Polar amplification throughout the interval from 3.56 to 2.2 Ma makes it likely that seasonal rather than perennial Arctic sea ice (5) was the norm, particularly during warm summer orbits [(61) and supplementary materials].

Conclusions

The sediments recovered at Lake El'gygytyn provide an exceptional window into environmental dynamics from a terrestrial high-latitude

site against which other Arctic and lower-latitude paleoenvironmental records can be compared. Although the geologic record has already informed us of extreme warmth at intervals in the middle Pliocene [(31) and references therein], Lake El'gygytyn provides a continuous time series of the timing of changes responsible for transforming a virtually ice-free forested Arctic into one capable of supporting major glacial cycles. Elevated warmth across cold and warm orbital cycles during the "41,000-year obliquity-dominated world" of the Pliocene and early Pleistocene is consistent with $p\text{CO}_2$ estimates of 280 to 400 ppm (2, 3, 62), but evidence of extreme polar amplification exceeds that simulated by many climate models. Mechanistic explanations for observed trends in temperature and precipitation have yet to be determined, but imply high sensitivity to CO_2 forcing. The delay of summer cooling in the terrestrial Arctic provides new constraints on Arctic climate and glacial evolution during the onset of NHG.

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Supplementary Materials

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REPORTS

Massive Dirac Fermions and Hofstadter Butterfly in a van der Waals Heterostructure

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van der Waals heterostructures constitute a new class of artificial materials formed by stacking atomically thin planar crystals. We demonstrated band structure engineering in a van der Waals heterostructure composed of a monolayer graphene flake coupled to a rotationally aligned hexagonal boron nitride substrate. The spatially varying interlayer atomic registry results in both a local breaking of the carbon sublattice symmetry and a long-range moiré superlattice potential in the graphene. In our samples, this interplay between short- and long-wavelength effects resulted in a band structure described by isolated superlattice minibands and an unexpectedly large band gap at charge neutrality. This picture is confirmed by our observation of fractional quantum Hall states at $\pm 5/3$ filling and features associated with the Hofstadter butterfly at ultrahigh magnetic fields.

The ability to tailor the properties of electronic devices is one of the landmark achievements of modern technology and motivates a sizable fraction of modern research in condensed matter physics. Just as crystal structure can determine the electronic properties of a material, artificial periodic superstructures can be used to modify the electronic band structure of existing materials (1). The band structure of pristine graphene consists of linearly dispersing energy bands, which touch at two degenerate “Dirac points.” This degeneracy is protected by the equivalence of the A and B triangular sublattices that make up the graphene honeycomb (2) and is responsible for graphene’s semimetallic behavior. Theory suggests that the electronic properties of graphene can be tuned via external periodic potentials: Long-wavelength superlattices have been predicted to lead to the formation of additional gapless Dirac points at finite energy (3), whereas atomic-scale modulations, by breaking the A-B sublattice symmetry, may turn graphene from a semimetal into a semiconductor (4). Experimental efforts to make high-mobility functional de-

vices based on band structure engineering, however, have been hindered by growth and nanofabrication limitations (5).

Recently, a new approach has become available through the use of hexagonal boron nitride (hBN) as a planar crystalline substrate. hBN is isostructural to graphene but has boron and nitrogen atoms on the A and B sublattices, leading to a large band gap (6). The weak interlayer van der Waals forces in both graphene and hBN permit the fabrication of multilayer heterostructures by sequential transfer of individual layers (7). During the transfer process, the angular alignment of the constituent crystals (θ) can in principle be controlled, but the graphene and hBN lattices retain their natural 1.8% mismatch (4). The frequency difference of the mismatched lattices leads to the formation of a moiré pattern with wavelength $\lambda(\theta)$ that can be much larger than the lattice constant (8, 9) (Fig. 1A).

The effect of the moiré on the graphene electronic structure can be decomposed into two parts (10). The moiré pattern results in a λ -scale modulation of the graphene-hBN coupling, forming a smooth superlattice potential. More subtly, the moiré also modulates the local asymmetry

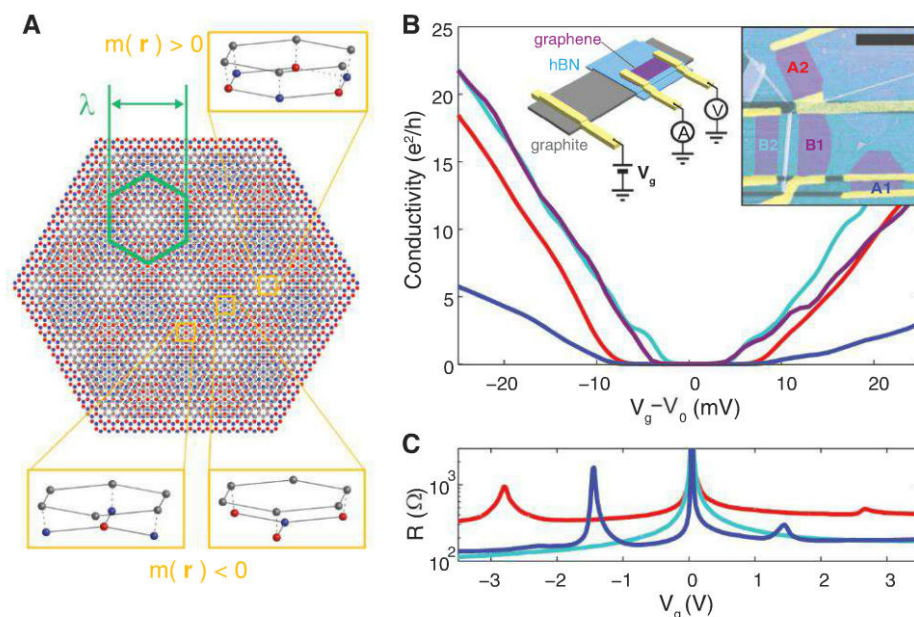


Fig. 1. Insulating states and superlattice minibands in a graphene-hBN heterostructure. (A) Schematic of the moiré pattern for graphene (gray) on hBN (red and blue), for zero misalignment angle and an exaggerated lattice mismatch of $\sim 10\%$. The moiré unit cell is outlined in green. Regions of local quasi-epitaxial alignment lead to opposite signs of the sublattice asymmetry, $m(r)$, in different regions (gray, carbon; red, boron; blue, nitrogen). (B) Low-temperature ($T = 150$ mK) conductivity near charge neutrality of four heterostructure devices (A1, A2, B1, and B2). The CNP offset $V_0 = 37, 37, 46,$ and 42 mV, respectively. Left inset: Measurement schematic. Right inset: False-color atomic force microscopy image. Scale bar, $3 \mu\text{m}$. (C) Resistance over a larger gate range. Finite-density resistance peaks indicate full filling of the lowest superlattice miniband in two of the four measured devices (A1 and A2) within the experimentally accessible density range.

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between the graphene sublattices induced by the difference in potential between boron and nitrogen atoms in the hBN. The resulting A-B potential difference in the graphene, parameterized in Fig. 1A as $m(\mathbf{r})$, oscillates across the superlattice unit cell (11), leading to nearly complete cancellation (10) upon spatial averaging. As we demonstrate, however, the absence of sublattice symmetry nonetheless has considerable experimental consequences for the electronic properties near the charge neutrality point.

We present measurements of four heterostructure devices consisting of a monolayer graphene flake on a 7-nm-thick hBN substrate (9), which itself sits on top of a graphite local gate (Fig. 1B). The proximal gate electrode serves both as an extremely flat substrate and to screen long-range potential fluctuations in the graphene (12), leading to high-quality devices with field-effect mo-

bilities of $\sim 100,000 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ and well-quantized quantum Hall plateaus at fields $B < \sim 100 \text{ mT}$ (9). In contrast to the majority of graphene devices—which, with few exceptions (12, 13), are semimetallic with zero-field minimum conductivity of $\sim 2e^2/h$ (where e is the electron charge and h is Planck's constant)—all four devices are strongly insulating near the overall charge neutrality point (CNP) (Fig. 1B). In addition, two devices show pronounced resistance peaks at finite density (Fig. 1C), situated symmetrically about the CNP. Notably, the devices showing additional resistance peaks also have the strongest insulating states.

We ascribe the satellite resistance peaks to the Bragg scattering of charge carriers by the superlattice when the lowest electron and hole minibands are fully occupied (8, 14–16). In graphene, as a consequence of the spin and valley degeneracies, full filling occurs at a density of four electrons

per superlattice unit cell, $n = 4n_0$, where $1/n_0 = \sqrt{3}\lambda^2/2$ is the unit cell area (Fig. 1A). Using the density at which the peaks are observed for devices A1 and A2, we estimate $\lambda_{A1} = 11$ to 13.5 nm and $\lambda_{A2} = 7.5$ to 9.5 nm , where the error is dominated by uncertainty in the value of the hBN dielectric constant (9). Such large values of λ , not much smaller than the theoretical maximum of 14 nm , imply nearly perfect rotational alignment of the graphene and hBN, with $\theta < 2^\circ$ for the two devices showing satellite peaks. Although we did not observe satellite peaks in devices B1 and B2, scanning tunneling topography measurements revealed moiré patterns with $\lambda_{B1} = 3.8 \text{ nm}$ and $\lambda_{B2} = 3.5 \text{ nm}$ (9), corresponding to close alignment with $\theta \approx 4^\circ$.

The moiré pattern offers a unique opportunity to study the elementary problem of a charged quantum particle moving under the simultaneous

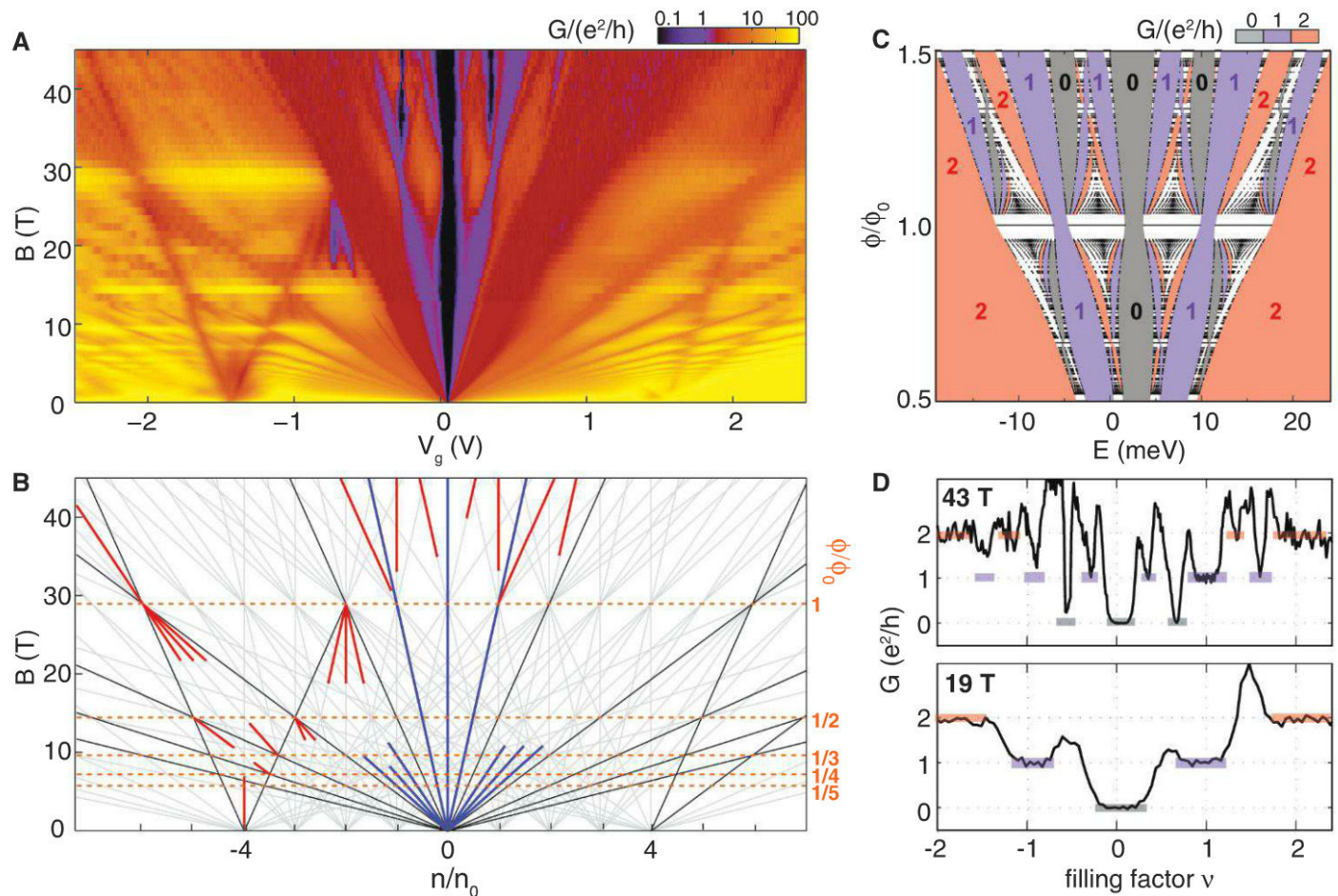


Fig. 2. Hofstadter butterfly. (A) Two-terminal magnetoconductance of device A1 up to 45 T. (B) In Wannier's theory (21), energy gaps in the Hofstadter spectrum are confined to linear trajectories $\phi/\phi_0 = (n/n_0 - s)/t$, where s and t are integers denoting the superlattice miniband filling index (33) and quantized Hall conductance of the gapped state, respectively. Gray lines indicate gaps for $-4 \leq s \leq 4$, with colored overlays indicating features observed in (A). Black, gaps requiring no broken symmetry; blue, broken-symmetry states for the central Landau fan; red, broken-symmetry states belonging to superlattice ($s \neq 0$) Landau fans. Gaps intersect at $\phi/\phi_0 = 1/q$, where q is an integer (orange); $\phi = \phi_0$ at 29 T. (C) Theoretical Hofstadter energy spectrum for the fully spin- and

sublattice-split $N = 0$ Landau level (9, 34). Black points indicate regions of dense energy bands; intervening spectral gaps are color-coded to the associated two-terminal conductance $G = 2$ (red), 1 (purple), and 0 (gray) in units of e^2/h . (D) Conductance traces within the $N = 0$ Landau level at $B = 43 \text{ T}$ (top) and $B = 19 \text{ T}$ (bottom). Shaded rectangles are color-coded to the expected two-terminal conductance from the Hofstadter model of (C). The emergence of Hofstadter minigaps, characterized by a nonmonotonic sequence of quantized conductance plateaus, is evident in the 43 T data, where $\phi > \phi_0$. At 19 T, the quantum Hall sequence is the standard monotonic $G = \nu e^2/h$. Peaks between plateaus are due to diffusive transport in this wide-aspect ratio device.

influence of a periodic potential and a magnetic field (17–20) in the normally inaccessible regime of more than one magnetic flux quantum (ϕ_0) per superlattice unit cell. In the absence of the superlattice, graphene is described at high fields by a set of discrete, highly degenerate Landau levels indexed by an integer N . The periodic potential splits the flat Landau level bands into “Hofstadter minibands” separated by a hierarchy of self-similar minigaps (18). Despite the intricate structure of the Hofstadter spectrum, the densities corresponding to the fractal minigaps follow simple linear trajectories as a function of magnetic field (21). Magnetoresistance data indeed show strong effects of the superlattice (Fig. 2A), including Landau fans originating from both the central and satellite zero-field resistance peaks. As recently demonstrated, the intersections between the central and satellite fans occur at $\phi = \phi_0/q$ (Fig. 2B), where ϕ is the magnetic flux per superlattice unit cell and q is a positive integer (15, 16). These intersections allow a second, independent method of measuring the unit cell area without reference to electrostatic parameters (9), giving $\lambda_{A1} = 12.9 \pm 0.2$ nm and $\lambda_{A2} = 9.2 \pm 0.1$ nm.

The full development of the Hofstadter butterfly, however, is most obvious in the regime $\phi/\phi_0 > 1$, which is challenging to access in monolayer graphene. Figure 2D shows the conductance within the $N = 0$ Landau level for two values of field corresponding to $\phi < \phi_0$ and $\phi > \phi_0$. At the higher field, the $N = 0$ Landau level is completely reconstructed, with a nonmonotonic sequence of conductance plateaus well-matched by tight binding calculations (9) of the Hofstadter butterfly spectrum in which phenomenological spin and sublattice symmetry-breaking terms have been included (Fig. 2C). The emergence of states with integer quantized conductance at noninteger filling of a single Landau level, severing the canonical relationship between quantized conductance and filling fraction, is the signature of the Hofstadter butterfly.

Equating the effect of the hBN substrate with that of a smooth superlattice potential explains many features of the experimental data, including the satellite resistance peaks and most features of the ultrahigh- B transport data. However, this fails to account for the insulating state observed at charge neutrality, which persists uninterrupted

from $B = 0$ to 45 T. To further explore the properties of this state, we used a low-temperature capacitance bridge to measure the capacitance of the graphene to the proximal graphite back gate (9, 22). Capacitance measurements probe the thermodynamic density of states, $\partial n/\partial \mu$; for our parallel plate geometry, the measured capacitance $C_{\text{meas}}^{-1} = C_{\text{geom}}^{-1} + (Ae^2 \partial n/\partial \mu)^{-1}$, where C_{geom} is the geometric capacitance and A is the sample area (23). Figure 3A shows magnetocapacitance data from a typical, semimetallic graphene-on-hBN device. The capacitance, and by extension the density of states, has a minimum at charge neutrality. As the field is increased, this minimum is replaced by a local maximum, signifying the formation of the zero-energy Landau level characteristic of massless Dirac fermions (24). Capacitance measurements of an insulating graphene device reveal very different behavior (Fig. 3B). No peak forms at the CNP at finite field, as can be seen from the dark vertical region centered at gate voltage $V_g = 41$ mV, indicating that the $N = 0$ Landau level is split into two finite-energy sublevels; in other words, a Landau level never forms at zero energy.

As we increase the field further (Fig. 3C), additional minima in capacitance appear at all integer filling factors ν —including those not belonging to the standard monolayer graphene sequence—indicating the emergence of exchange-driven broken-symmetry states (25). Capacitance minima associated with fractional quantum Hall states appear (Fig. 3D) at all multiples of $1/3$ for $-2 < \nu < 2$. Notably, this sequence includes robust features at $\nu = \pm 5/3$ not observed in previous studies (26, 27) of semimetallic monolayer graphene. These states are thought to be described by a fully spin- and sublattice-polarized Laughlin wave function (28, 29); their absence in semimetallic graphene is attributed (26, 30) to the low energy cost of exciting charge carriers to the unoccupied, energetically equivalent sublattice. Our observation of the $\pm 5/3$ states suggests that sublattice symmetry is broken in our graphene-hBN heterostructures.

In the Dirac equation description of graphene, sublattice symmetry breaking can be parameterized by a mass. In our heterostructures, this mass term $m(\mathbf{r})$ is expected to oscillate across the moiré unit cell (Fig. 1A). Notably, the low-density phenomenology of our insulating graphene at low fields ($\phi \ll \phi_0$)—including the insulating gap, the absence of a zero-energy Landau level, and the observation of the $\nu = \pm 5/3$ states—can be captured by a Dirac equation with a spatially uniform global effective mass, m^* . The resulting Hamiltonian describing physics in the vicinity of the K (K') point is

$$\begin{aligned} \hat{H} &= v_F \boldsymbol{\sigma} \cdot \mathbf{p} + m^* v_F^2 \hat{\sigma}_z \\ &= \begin{pmatrix} \pm m^* v_F^2 & v_F(p_x - i p_y) \\ v_F(p_x + i p_y) & \mp m^* v_F^2 \end{pmatrix} \quad (1) \end{aligned}$$

where v_F is the Fermi velocity, $\mathbf{p} = (p_x, p_y)$ is the momentum in the graphene plane, $\boldsymbol{\sigma} = (\sigma_x, \sigma_y)$,

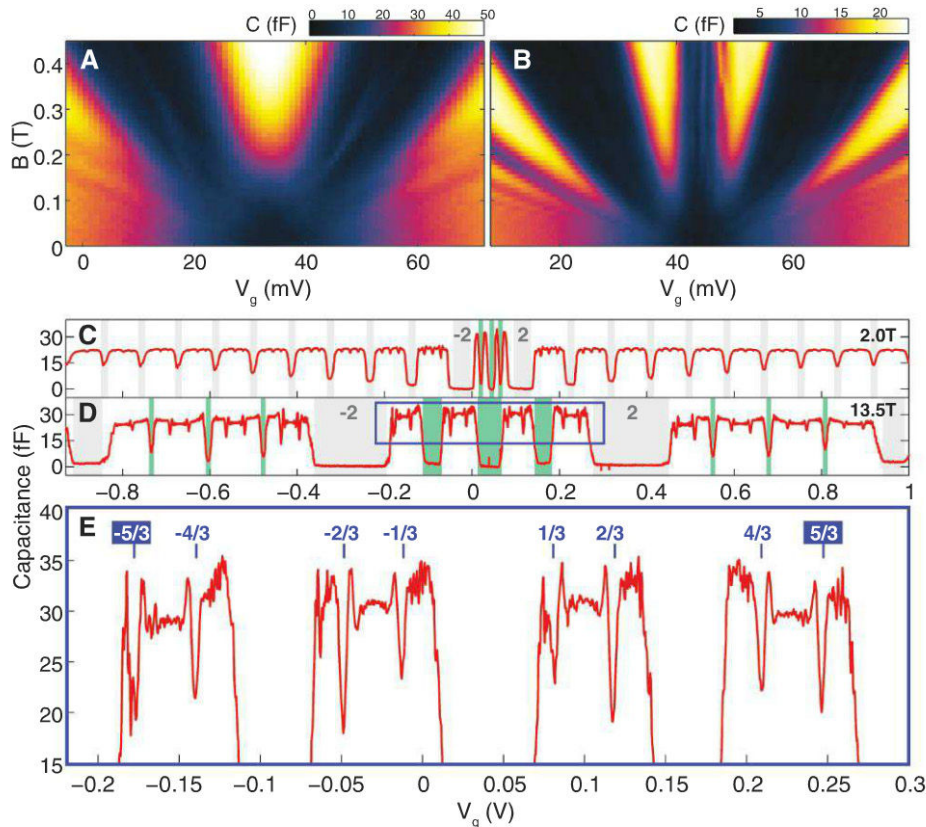


Fig. 3. Magnetocapacitance of semimetallic and insulating graphene devices. (A) Capacitance of a typical semimetallic graphene device. The zero-energy Landau level forms at ~ 0.15 T, appearing as a local maximum at the CNP ($V_g = 34.5$ mV). (B) Capacitance of an insulating graphene device (B2) under similar conditions. In contrast to (A), the density of states is at a local minimum for all fields at charge neutrality ($V_g = 44$ mV). (C and D) Capacitance of device B2 at $B = 2.0$ T (C) and 13.5 T (D). Cyclotron gaps are shaded gray ($\nu = \pm 2$ labeled) and broken-symmetry gaps are shaded green. (E) Fractional quantum Hall (FQH) states in the $N = 0$ Landau level. The incompressible features at $\nu = \pm 5/3$ are of similar strength to the other FQH states.

and the Pauli matrices $\{\sigma_i\}$ operate in the basis of the two sublattices. The resulting energy spectrum at zero magnetic field,

$$E(\mathbf{p}) = \pm \sqrt{v_F^2 |\mathbf{p}|^2 + (m^* v_F^2)^2} \quad (2)$$

features a band gap $\Delta = 2m^* v_F^2$ at charge neutrality. In a quantizing magnetic field, the Landau level spectrum is given by

$$E_N = \pm \sqrt{2(\hbar v_F)^2 |N|/\ell_B^2 + (m^* v_F^2)^2} \quad (3)$$

where $\hbar = h/2\pi$ and $\ell_B = \sqrt{\hbar/(eB)}$ is the magnetic length (9). The mass term does not lift the Landau level degeneracy for $|N| > 0$; this is reflected in our data (Fig. 3C) by the observation of symmetry breaking in the higher Landau levels only at higher magnetic fields, presumably due to exchange interactions, as in semimetallic graphene (25). However, the splitting of the $N = 0$ Landau level into two sublattice-polarized branches at $E_0 = \pm m^* v_F^2$ is consistent with the persistent gap at charge neutrality, as well as the observation of the $\pm 5/3$ fractional quantum Hall states.

The observation of band gaps in samples with long-wavelength moiré patterns is not likely to be coincidental. Naïvely, $m(\mathbf{r})$ nearly vanishes upon spatial average, calling into question whether a mismatched hBN substrate can open a measurable gap. Indeed, predictions for how the global parameter m^* depends on the microscopic structure of $m(\mathbf{r})$ vary by several orders of magnitude depending on theoretical assumptions (4, 10, 31, 32). To quantitatively assess the connection between

λ and m^* , we measured the band gap using the temperature dependence of the charge-neutrality point conductivity (Fig. 4, A and B) and independently measured the width in gate voltage of the insulating state, which places an upper bound on the chemical potential difference across the band gap (9). Figure 4C shows a correlation between moiré wavelength and the measured gaps, which suggests that the interaction of the graphene flake with the closely aligned hBN substrate is responsible for the insulating behavior. Single-particle theories of graphene on hBN (10, 31) predict $\Delta \approx 5$ to 10 meV for zero misalignment angle, for which the gap is expected to be maximal. Our measured band gaps exceed these predictions even for non-zero twist. In addition, the discrepancy between the two methods for gap determination implies that Δ depends on chemical potential, suggesting that many-body interactions may play a role in enhancing Δ (32). However, quantitative comparison with the predicted power-law dependence of $\Delta(\lambda)$ will require more extensive data.

The ability to engineer a nonzero band mass in graphene has far-reaching implications for future experimental efforts. The possibility of an alignment-dependent mass may require a reinterpretation of experiments involving graphene-hBN heterostructures, even as it engenders new opportunities for the design of electronic devices. The gapped spectrum and the tunability of the associated effective mass provide a useful tool in nanoengineering based on electrostatic confinement, as well as a new design parameter in the study of many-body effects in monolayer graphene.

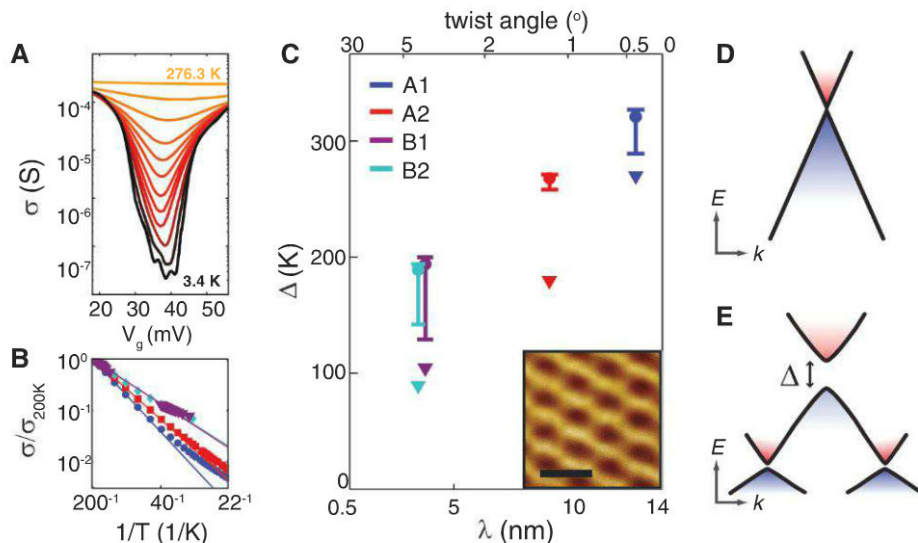


Fig. 4. Energy gaps of the zero-field insulator. (A) Conductivity of the insulator device A1 at a series of temperatures. (B) Arrhenius plot of σ_{CNP} for all four devices. Lines are fits to the simply activated form $\sigma_{\text{CNP}}(T) \propto \exp(-\Delta/2T)$, giving $\Delta_{A1} = 321$ K, $\Delta_{A2} = 268$ K, $\Delta_{B1} = 194$ K, and $\Delta_{B2} = 189$ K. (C) Correlation of observed band gaps with moiré wavelength λ . Circles denote thermal activation gap (Fig. 2B); error bars are estimated from the uncertainty in the range of simply activated behavior. Inverted triangles denote width in gate voltage of the insulating state. λ is extracted from the period of the Hofstadter oscillations for A1 and A2, and from STM measurements for B1 and B2. Inset: Scanning tunneling topography image of B2 (scale bar, 5 nm). (D) Schematic band structure for semimetallic graphene, showing the linear dependence of energy E on two-dimensional wave vector $k = |\mathbf{p}|/\hbar$. (E) Schematic band structure for an insulating graphene-hBN heterostructure, showing the band gap and moiré minibands.

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Engineering Coherence Among Excited States in Synthetic Heterodimer Systems

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The design principles that support persistent electronic coherence in biological light-harvesting systems are obscured by the complexity of such systems. Some electronic coherences in these systems survive for hundreds of femtoseconds at physiological temperatures, suggesting that coherent dynamics may play a role in photosynthetic energy transfer. Coherent effects may increase energy transfer efficiency relative to strictly incoherent transfer mechanisms. Simple, tractable, manipulable model systems are required in order to probe the fundamental physics underlying these persistent electronic coherences, but to date, these quantum effects have not been observed in small molecules. We have engineered a series of rigid synthetic heterodimers that can serve as such a model system and observed quantum beating signals in their two-dimensional electronic spectra consistent with the presence of persistent electronic coherences.

The discovery of long-lived coherence between the singly excited electronic states of photosynthetic antenna complexes has necessitated both a rethinking of energy transfer in biological systems and, more broadly, a reconsideration of the role of the surrounding environment and of coherent dynamics in the condensed phase (*1*). These “persistent coherences” unexpectedly outlive coherences between the constituent singly excited states and the electronic ground

state. An ever-growing body of work demonstrates the persistence of electronic coherence during energy transfer in a multitude of antenna complexes across broad phylogenetic boundaries (*2–5*), and coherence within the Fenna-Matthews-Olson complex (FMO) has been shown to be robust to vibronic and structural modifications (*6*). This remarkable generality suggests that persistent electronic coherences may be a general property of any system of densely packed chromophores arranged in a nearly static relative geometry and coupled to a common bath.

Despite numerous observations of persistent electronic coherence in photosynthetic systems, no clear microscopic mechanism for the survival

of these coherences has been experimentally verified. Theoretical efforts to dissect the atomistic mechanism are complicated by the size and complexity of photosynthetic light-harvesting systems. Consequently, many competing models have been introduced to explain the observed quantum beating. These models invoke a broad range of physical mechanisms, including vibrational coherences (*7, 8*), vibronic excitons (*9*), nonadiabatic couplings (*10*), correlated protein motion (*11*), nonsecular coupling between coherence and population (*12*), and long-range dielectric fluctuations (*13*). In the present work, we provide evidence that a synthetic small molecule can reproduce the long-lived quantum coherence phenomenon observed in photosynthetic light-harvesting systems.

A synthetic system that supports long-lived electronic coherence observable with two-dimensional (2D) electronic spectroscopy has only a few requirements—a pair of chromophores, a rigid linker that constrains the relative orientation of the chromophores, and an appropriate solvent bath—yet, long-lived electronic coherences have still only been observed in polymeric systems. In general, Förster resonance energy transfer is sufficient for describing incoherent energy transfer between small molecules in dilute solution, but a fluctuating system of two chromophores held in a fixed relative orientation provides an opportunity to observe coherent dynamics. Such a system also provides a greatly simplified model for investigating the role and mechanism of coherent energy transfer in multichromophoric systems, which remain largely unknown because of the complexity of the 2D spectra of photosynthetic antennae.

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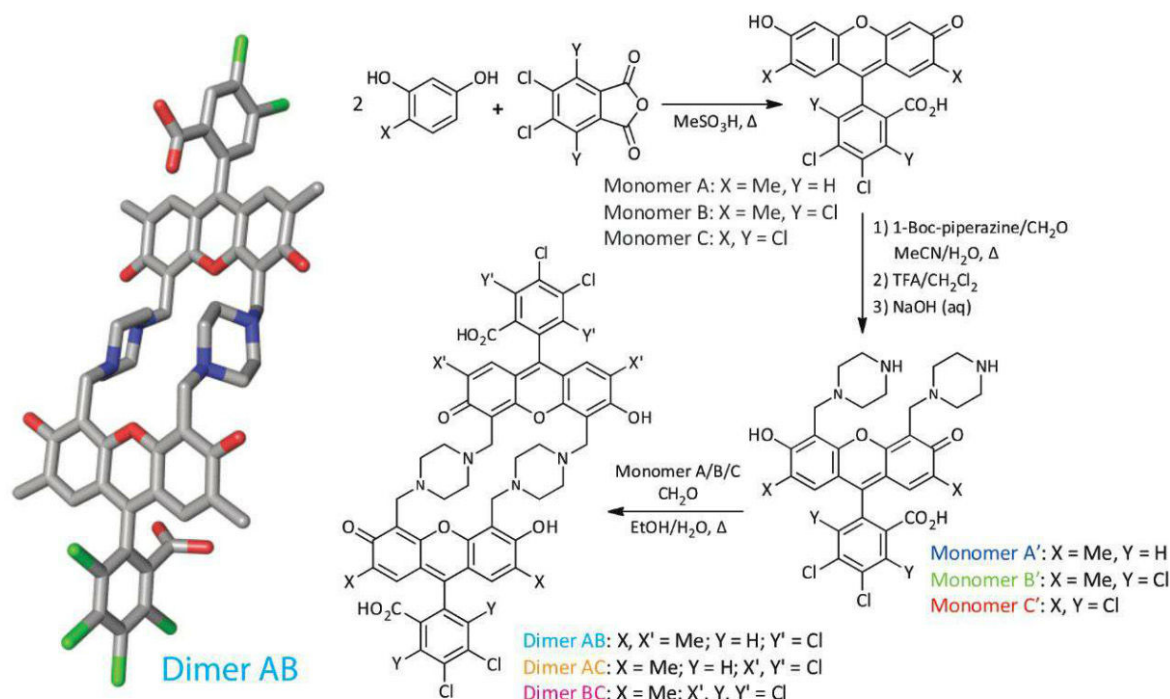


Fig. 1. Engineering small molecules to support long-lived electronic coherence. (Left) The optimized geometry of dimer AB obtained by using the B3LYP functional with the 6-311G(d,p) basis set. (Right) Synthesis of halofluorescein dimers. Boc, t-butyloxycarbonyl; TFA, trifluoroacetic acid.

Although excitonic splitting in a homodimer of strongly coupled chromophores yields two nondegenerate excited states, the persistence of electronic coherence in such molecular aggregates is a trivial consequence of the shared nature of the states, called exchange narrowing (14). We investigated the less straightforward case of a rigid, weakly coupled heterodimer, which is a popular subject of theoretical work (15). Restricted relative motion of the monomers should correlate energetic fluctuations in such a system, thereby slowing dephasing of the coherence between the two mostly localized electronic excited states. We have prepared a series of such dimers (Fig. 1) and acquired a series of 2D electronic spectra of each dimer in solution at room temperature in order to investigate the sub-picosecond dynamics of quantum coherences in these systems under ambient conditions. These heterodimers also present a platform on which to build an understanding of 2D electronic spectra, bridging the gap between simple laser dyes and complex biological systems (16). Although a considerable body of work has been published on the ultrafast spectra of metal-bridged molecular homodimers (17, 18), the dynamics of these systems are strongly influenced by charge-transfer states, which complicate interpretation of oscillatory signals (19). Furthermore, the tunability of the energy gap of our heterodimers provides both a tool for studying the impact of coherence on energy transfer and an internal reference for unambiguously assigning quantum beats to electronic coherences. Molecular heterodimers have recently been the subject of theoretical studies (15, 20), but complementary experimental results are essential to our understanding of energy transfer and nonlinear spectroscopy.

We chose to use variably substituted fluoresceins as monomers because the visible absorption spectra are relatively narrow and can be tuned by means of halogenation to fall within the bandwidth of our laser pulse. Furthermore, aminoalkylation of the 4' and 5' positions of fluorescein via Mannich condensation is well documented (21), providing a framework for "bidentate" dimerization through use of two equivalents of an appropriate unconjugated diamine and formaldehyde (Fig. 1). This approach requires that the 2' and 7' positions of each monomer be substituted to prevent reaction at these α -keto positions as well. Accordingly, we chose 5,6-dichloro-2',7'-dimethylfluorescein (monomer A), 4,5,6,7-tetrachloro-2',7'-dimethylfluorescein (monomer B), and 4,5,6,7-tetrachloro-2',7'-dichlorofluorescein (monomer C) for the monomers and prepared them according to a literature method (22).

Piperazine, an inexpensive cyclic diamine, proves to be an ideal linker for this system. The rigidity of piperazine not only restricts the relative motion of the linked monomers to suppress dephasing and inhomogeneity but also prevents intramolecular cyclization reactions from occurring at the unreacted amine after the initial condensation reaction. Dialkylated piperazine

also separates the dipoles of the monomers by a distance of 10 Å, which is short enough to introduce a small degree of electronic coupling but long enough to keep each exciton mostly localized on a single chromophore. In our synthetic scheme, Mannich condensation of two equivalents of 1-Boc-piperazine with formaldehyde and monomer A, B, or C, and subsequent deprotection, yields aminoalkylated monomer A', B', or C'. Macrocyclization of this intermediate with another monomer via a second Mannich reaction affords the dimer in moderate yield. Homodimers can be prepared simply via a one-pot Mannich reaction with one equivalent each of piperazine and monomer A, B, or C and excess paraform-

aldehyde. No evidence of trimers or higher-order coupling products was found in mass spectra of the crude or purified products from either method.

Samples of the dimers were prepared in ethanol with 1% 0.1 M aqueous NaOH and diluted to an optical density of 0.2 in 200 μ m, corresponding to a concentration of ~ 0.1 mmol. Formation of intermolecular dimers and trimers under these conditions should be negligible (23). Normalized linear absorption and fluorescence spectra (excitation at 510 nm) of each sample are shown in Fig. 2, with the spectrum of the laser pulse used for the 2D experiments overlaid. Linear spectra of the dimers were also taken at 77 K in order to assess the strength of electronic coupling be-

Fig. 2. Linear absorption and fluorescence spectra of fluorescein heterodimers. Linear absorption (solid lines) and fluorescence (dashed lines) spectra of dimers AB (blue), AC (orange), and BC (magenta) taken in ethanol with NaOH. The laser pulse used to acquire 2D spectra is shown in gray.

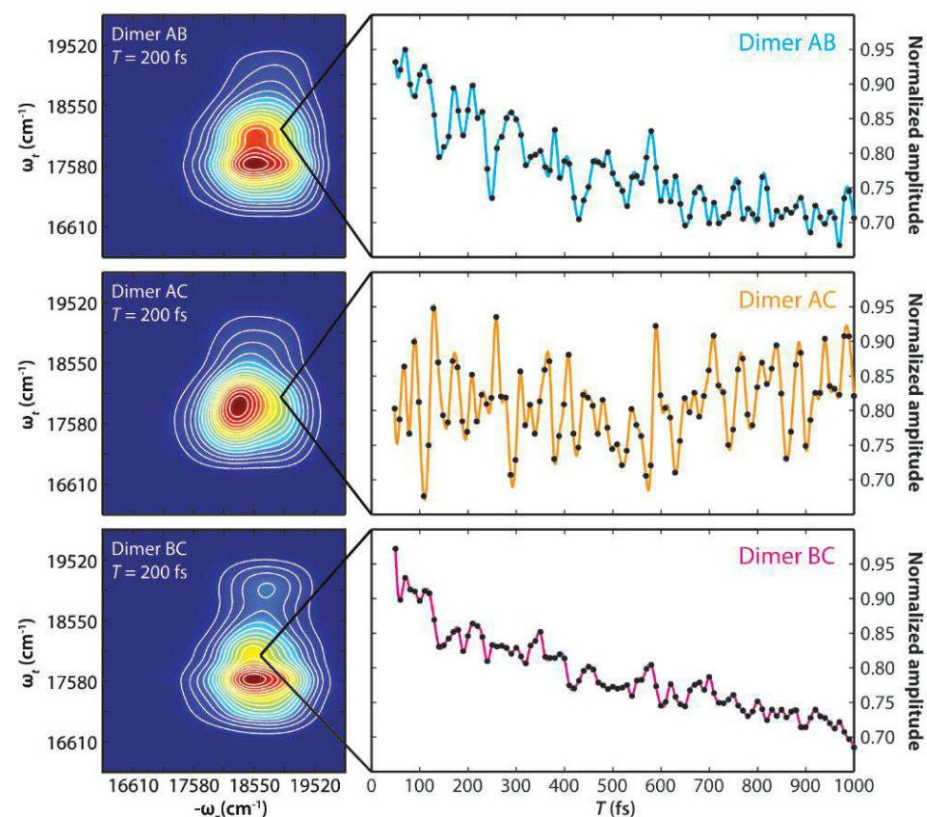
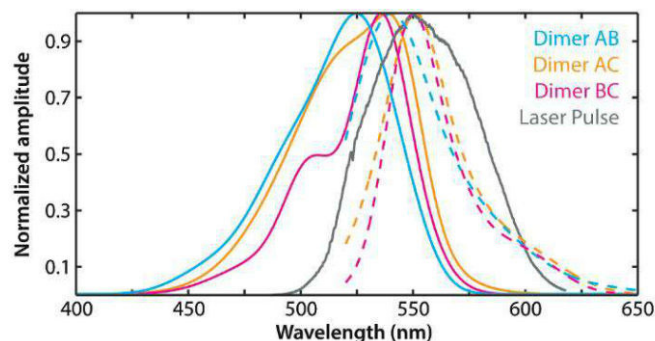


Fig. 3. Cross peaks in 2D spectra show complex oscillations. Absolute-value 2D spectra of dimers AB, AC, and BC acquired at $T = 200$ fs are shown with linearly spaced contour lines. Raw time traces taken from the indicated crosspeaks for $T = 50$ to 1000 fs are plotted to the right, with data points shown in time steps of 10 fs (interpolated traces shown as colored lines).

tween the monomers, but even at cryogenic temperature, the exciton peaks are not individually resolved, indicating that the coupling is weak. The absorption spectra of monomers A', B', and C' (fig. S1) were used to approximate the energies of the electronic transitions in the dimers, giving 524.1, 538.3, and 544.2 nm, respectively. The shapes of these spectra further confirm that intermolecular dimers and trimers are not present in significant concentrations.

Two-dimensional electronic spectroscopy has been used to observe quantum beats arising from electronic coherences and quantify their dephasing rates in a number of photosynthetic antenna complexes, including the FMO complex (1), phycobiliproteins (3), light-harvesting complex 2 (LH2) (4), and LHCII (2). This method is particularly suited to such experiments because it separates signals arising from coherences into specific, predictable locations in a frequency-frequency spectrum (24). In this four-wave mixing technique, which has been thoroughly described elsewhere (25–27), a pair of phase-locked, ultrashort laser pulses interacts with the sample, yielding a population state or a coherence between singly excited states. The system is allowed to evolve for a time T (the waiting time) before a third pulse interacts with the sample, resulting in the emission of the third-order signal. The 2D spectra presented here can be considered to plot “energy in” along the coherence frequency axis (ω_c , horizontal axis) and “energy out” along the rephasing frequency axis (ω_r , vertical axis). Phase-matching considerations dictate the use of negative coherence frequencies (“ $-\omega_c$ ” rather than “ ω_c ”). A series of 2D spectra taken at different waiting

times gives ultrafast temporal resolution of spectral features, revealing both incoherent (exponential) relaxation dynamics and coherent (oscillatory) evolution in peak shapes and amplitudes. The lifetime of coherences can then be measured as the damping rate of such oscillatory signals (28).

Series of 2D spectra were acquired at waiting times $T = 50$ to 1000 fs in steps of 10 fs for each dimer. Control experiments were also performed with monomers A', B', and C' individually; equimolar mixtures of pairs of monomers A', B', and C'; a homodimer (dimer BB); and a solvent blank. Absolute value 2D spectra for each heterodimer taken at $T = 200$ fs are shown in Fig. 3, whereas spectra of the mixtures of monomers are shown in fig. S2. The features in the $T = 200$ fs spectra of the dimers are significantly broader along $-\omega_c$ than their respective linear absorption spectra as a result of fast relaxation during the coherence time. The majority of the signal appears well below the diagonal, due in part to this fast Stokes shift occurring during early waiting times. Perhaps more important, however, is the spectral position of the laser pulse, which is centered near the emission frequencies of the dimers (Fig. 2) with substantial bandwidth extending further into the red, favoring stimulated emission to vibrationally excited states within the electronic ground state manifold (29). In 2D electronic spectroscopy, we detect only signals within our laser bandwidth, so overlapping with both the absorption and emission spectra ensures that we retain a signal throughout the Stokes shift.

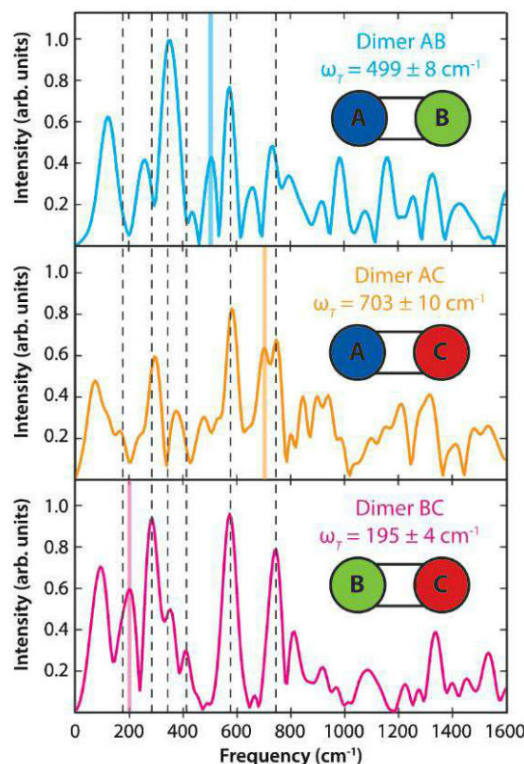
Time traces for each dimer were taken by integrating the absolute value signal over a 30 by 30 cm^{-1} square centered at a value of ω_c equal to

the absorption maximum of the higher-energy aminoalkylated monomer and at a value of ω_r equal to the absorption maximum of the lower-energy monomer. For comparison, traces were taken from the spectra of the corresponding equimolar mixtures of monomers (monomers A' and B' for dimer AB, monomers A' and C' for dimer AC, and monomers B' and C' for dimer BC) at the same spectral locations. Each trace was fit to the sum of two exponential decays, and Fourier transforms of the apodized residuals are plotted in Fig. 4. These power spectra show the relative contributions of the frequencies that produce the beating found in these traces. Although ethanol has a strong Raman mode at 883 cm^{-1} (30), this frequency is observed only in the spectra of the solvent blank, demonstrating that the resonant signal from all samples overwhelms the nonresonant solvent contribution in these experiments. Thus, the frequencies shown in Fig. 4 arise exclusively from the vibrational and electronic coherences of the dimers.

To facilitate assignment of peaks in these power spectra, the same analysis was performed on the spectra of the monomers (fig. S3). Between 150 and 1000 cm^{-1} , strong peaks were found at 275, 342, and 410 cm^{-1} in monomer A'; 342, 414, 581, and 737 cm^{-1} in monomer B'; and 175, 290, 573, and 750 cm^{-1} in monomer C' (the higher frequency peaks in the monomer spectra are not included for the sake of simplicity). These frequencies are indicated with dashed lines in Fig. 4, accounting for all but one of the frequencies observed in the spectra of the dimers. Because these peaks are all present in single-chromophore spectra, they are assigned to the vibrational modes of their respective monomers. These frequencies also account for most of the peaks observed in the generally noisy spectra of the mixtures of monomers (fig. S4).

An additional peak in the power spectrum of the trace taken from the spectra of dimer AB that is not present in the mixture of monomers A' and B' appears at 500 cm^{-1} . This result is consistent across three data sets acquired on different days with slightly different laser pulses. A least-squares fit of the residual trace to the sum of five damped sinusoids (fig. S5) gives a frequency of $499 \pm 8 \text{ cm}^{-1}$. This frequency is in excellent agreement with the 503 cm^{-1} difference between the absorption maxima of monomers A' and B' (Fig. 4, solid blue line), as is expected for a beat arising from electronic coherence within the dimer. Because of the large number of fitting parameters, however, the error in the dephasing time obtained from this fit is greater than the dephasing time itself. Therefore, an approximate dephasing time of 90 fs was obtained from the linewidth of the peak in the power spectrum, which because of the apodization process represents a lower bound. In contrast, for a fixed waiting time the signal decays completely within 40 fs of coherence time, giving a dephasing (e -folding) time of less than 15 fs for coherences between the ground state and the individual excited states.

Fig. 4. Power spectra of cross peak oscillations reveal persistent electronic coherence. Fourier transforms of waiting-time traces were taken from the lower crosspeaks of the 2D spectra of dimers AB (top), AC (middle), and BC (bottom). The energy gaps between the absorption maxima of the aminoalkylated monomers are shown as solid colored lines (monomers A' and B', blue; monomers A' and C', orange; monomers B' and C', magenta), whereas peaks assigned to vibrational coherences are indicated with dashed black lines.



As observed in FMO, coherences between singly excited electronic states persist longer than the coherences between the ground state and the individual excited states by an order of magnitude (28), suggesting that the energetic fluctuations responsible for dephasing each exciton are correlated.

A similar pattern is observed in the power spectrum of the trace taken from the spectra of dimer AC. Here, the prominent 693 cm^{-1} peak in the transform is absent from the transform from the control experiments. Although a nearby feature appears in the power spectrum from the corresponding mixture of monomers (fig. S4), this feature is below the noise floor of the transform, which we define as 1.5 times the mean value of the spectrum. The energy gap inferred from the monomers A' and C' absorption maxima is 705 cm^{-1} (Fig. 4, solid magenta line), which again is in excellent agreement with the beat frequency observed in the 2D spectra. This method of estimating the energy gap between excited states inherently neglects any shifts that may occur because of coupling between electronic states. The fit to this trace gives a frequency of $703 \pm 10\text{ cm}^{-1}$, and the dephasing time obtained from the linewidth of the peak in the power spectrum is 60 fs. Last, the power spectrum of dimer BC shows a strong peak at 199 cm^{-1} , which the fit to the trace refines to $195 \pm 4\text{ cm}^{-1}$, again matching the inferred energy gap of 201 cm^{-1} . The dephasing time for this peak is also 60 fs.

The time traces of the off-diagonal features in the spectra of the dimers exhibit oscillatory behavior associated with coherent evolution at frequencies corresponding to the respective transition energy gaps, whereas traces taken at identical spectral coordinates in the spectra of the corresponding monomers demonstrate only vibrational oscillations. This disparity allows us to assign these quantum beats to coherences between the electronic excited states of the dimers. The synthetic ease with which we control the energy gap between the monomers allows us to follow the frequency of the beating signal between three different dimers, further demonstrating the electronic nature of these oscillations.

By replicating in a simple synthetic system the long-lived electronic coherence observed in biological systems, we confirm the generality of this phenomenon and introduce a framework for dissecting the complex information contained in 2D spectra of natural multichromophoric systems. In creating the first model system for such effects, we have identified that proximity, fixed orientation, and electronic coupling are sufficient to support persistent coherence. This system will allow complementary experimental and theoretical approaches to fully understand the role of quantum coherence in chemical dynamics. Furthermore, this work lays the groundwork for the development of artificial energy-transfer devices that exploit the improved transfer efficiency obtained from the synergy between coherent and incoherent dynamics.

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Supplementary Materials

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Materials and Methods
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Direct Imaging of Covalent Bond Structure in Single-Molecule Chemical Reactions

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Observing the intricate chemical transformation of an individual molecule as it undergoes a complex reaction is a long-standing challenge in molecular imaging. Advances in scanning probe microscopy now provide the tools to visualize not only the frontier orbitals of chemical reaction partners and products, but their internal covalent bond configurations as well. We used noncontact atomic force microscopy to investigate reaction-induced changes in the detailed internal bond structure of individual oligo-(phenylene-1,2-ethynylene)s on a (100) oriented silver surface as they underwent a series of cyclization processes. Our images reveal the complex surface reaction mechanisms underlying thermally induced cyclization cascades of enedienes. Calculations using ab initio density functional theory provide additional support for the proposed reaction pathways.

Understanding the microscopic rearrangements of matter that occur during chemical reactions is of great importance for catalytic mechanisms and may lead to greater efficiencies in industrially relevant processes (1, 2). However, traditional chemical structure characterization methods are typically limited to

ensemble techniques where different molecular structures, if present, are convolved in each measurement (3). This limitation complicates the determination of final chemical products, and often it renders such identification impossible for products present only in small amounts. Single-molecule characterization techniques such as scanning tun-

neling microscopy (STM) (4, 5) potentially provide a means for surpassing these limitations. Structural identification using STM, however, is limited by the microscopic contrast arising from the electronic local density of states (LDOS), which is not always easily related to chemical structure. Another important subnanometer-resolved technique is transmission electron microscopy (TEM). Here, however, the high-energy electron beam is often too destructive for organic molecule imaging. Recent advances in tuning fork-based noncontact atomic force microscopy (nc-AFM) provide a method capable of nondestructive subnanometer spatial resolution (6–10). Single-molecule images obtained with this technique are reminiscent of wire-frame chemical structures and even allow differences in chemical bond order to be identified (10). Here we show that it is possible to resolve with nc-AFM the intramolecular structural changes and bond rearrangements associated with complex surface-supported cyclization cascades, thereby revealing the microscopic processes involved in chemical reaction pathways.

Intramolecular structural characterization was performed on the products of a thermally induced enediyne cyclization of 1,2-bis(2-ethynylphenyl)ethynylbenzene (**1**). Enediynes exhibit a variety of radical cyclization processes known to compete with traditional Bergman cyclizations (11, 12), thus often rendering numerous products with complex structures that are difficult to characterize using ensemble techniques (13). To directly image these products with subnanometer spatial resolution, we thermally activated the cyclization reaction on an atomically clean metallic surface under ultrahigh vacuum (UHV). We used STM and nc-AFM to probe both the reactant and final products at the single-molecule level. Our images reveal how the thermally induced complex bond rearrangement of **1** resulted in a variety of unexpected products, from which we have obtained a detailed mechanistic picture—corroborated by *ab initio* density functional theory (DFT) calculations—of the cyclization processes.

We synthesized 1,2-bis(2-ethynylphenyl)ethynylbenzene (**1**) through iterative Sonogashira cross-coupling reactions (scheme S1) (14). We deposited **1** from a Knudsen cell onto a Ag(100) surface held at room temperature under UHV. Molecule-decorated samples were transferred to a cryogenic imaging stage ($T \leq 7$ K) before and after under-

going a thermal annealing step. Cryogenic imaging was performed both in a home-built $T = 7$ K scanning tunneling microscope and in a qPlus-equipped (15, 16) commercial Omicron LT-STM/AFM at $T = 4$ K. nc-AFM images were recorded by measuring the frequency shift of the qPlus resonator while scanning over the sample surface in constant-height mode. For nc-AFM measurements, the apex of the tip was first functionalized with a single CO molecule (6). To assess the reaction pathway energetics, we performed *ab initio* DFT calculations within the local density approximation (17) using the GPAW (Grid-based Projector Augmented Wavefunction) code (18, 19).

Figure 1A shows a representative STM image of **1** on Ag(100) before undergoing thermal annealing. The adsorbed molecules each exhibited three maxima in their LDOS at positions suggestive of the phenyl rings in **1** (Fig. 1A). Annealing the molecule-decorated Ag surface up to 80°C left the structure of the molecules unchanged. Annealing the sample at $T \geq 90^\circ\text{C}$, however, induced a chemical transformation of **1** into distinctively different molecular products (some molecular desorption was observed). Figure 1B shows an STM image of the surface after annealing at 145°C for 1 min. Two of the reaction products can be seen in this image, labeled as **2** and **3**. The structures of the products are unambiguously distinguishable from one another and from the starting material **1**, as shown in the close-up STM images of the most common products **2**, **3**, and **4** in Fig. 2, B to D. The observed product ratios are $2:3:4 = (51 \pm 7\%):(28 \pm 5\%):(7 \pm 3\%)$, with the remaining products comprising other minority monomers as well as fused oligomers (fig. S2) (14).

Detailed subnanometer-resolved structure and bond conformations of the molecular reactant **1** and products (**2**, **3**, and **4**) were obtained by performing nc-AFM measurements of the molecule-decorated sample both before and after annealing at $T \geq 90^\circ\text{C}$. Figure 2E shows a nc-AFM image of **1** before annealing. In contrast to the STM image (Fig. 2A) of **1**, which reflects the diffuse electronic LDOS of the molecule, the AFM image reveals the highly spatially resolved

internal bond structure. A dark halo observed along the periphery of the molecule is associated with long-range electrostatic and van der Waals interactions (6, 20). The detailed intramolecular contrast arises from short-ranged Pauli repulsion, which is maximized in the regions of highest electron density (20). These regions include the atomic positions and the covalent bonds. Even subtle differences in the electron density attributed to specific bond orders can be distinguished (10), as evidenced by the enhanced contrast at the positions of the triple bonds within **1**. This effect is to be distinguished from the enhanced contrast observed along the periphery of the molecule, where spurious effects (e.g., because of a smaller van der Waals background, enhanced electron density at the boundaries of the delocalized π -electron system, and molecular deviations from planarity) generally influence the contrast (10, 20).

Figure 2, F to H, shows subnanometer-resolved nc-AFM images of reaction products **2**, **3**, and **4** that were observed after annealing the sample at $T > 90^\circ\text{C}$. The structure of these products remained unaltered even after further annealing to temperatures within the probed range from 90° to 150°C (150°C was the maximum annealing temperature explored in this study). The nc-AFM images reveal structural patterns of annulated six-, five-, and four-membered rings. The inferred molecular structures are shown in Fig. 2, J to L. Internal bond lengths measured by nc-AFM have previously been shown to correlate with Pauling bond order (10), but with deviations occurring near a molecule's periphery, as described above. As a result, we could extract clear bonding geometries for the products (Fig. 2, J to L), but not their detailed bond order. The subtle radial streaking extending from the peripheral carbon atoms suggests that the valences of the carbon atoms are terminated by hydrogen (20). This is in agreement with molecular mass conservation for all proposed product structures and indicates that the chemical reactions leading to products **2**, **3**, and **4** are exclusively isomerization processes (21).

Our ability to directly visualize the bond geometry of the reaction products (Fig. 2, F to H)

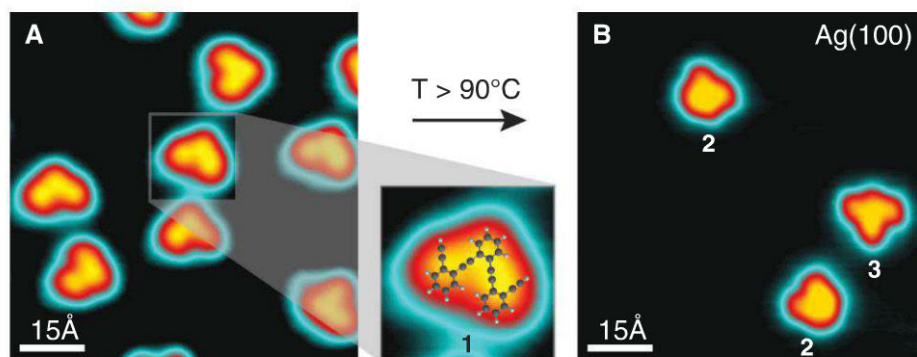


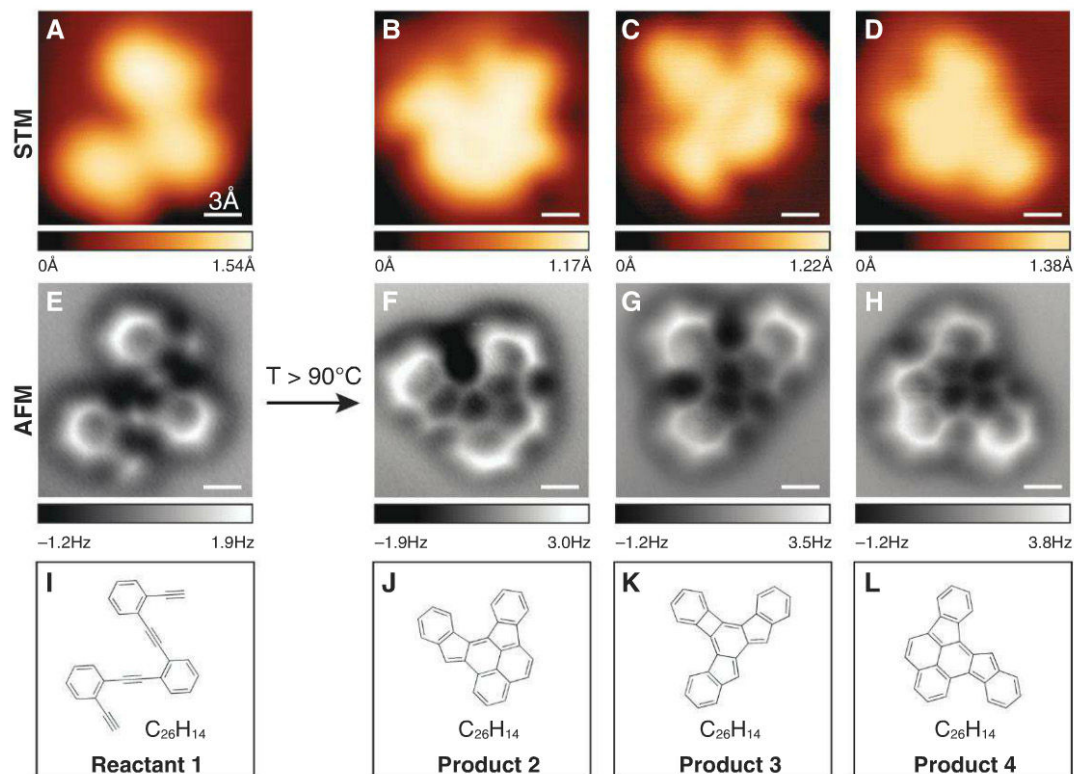
Fig. 1. STM images of a reactant-decorated Ag(100) surface before and after thermally induced cyclization reactions. (A) Constant-current STM image of **1** as deposited on Ag(100) ($I = 25$ pA, $V = 0.1$ V, $T = 7$ K). A model of the molecular structure is overlaid on a close-up STM image. (B) STM image of products **2** and **3** on the surface shown in (A) after annealing at $T = 145^\circ\text{C}$ for 1 min ($I = 45$ pA, $V = 0.1$ V, $T = 7$ K).

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Fig. 2. Comparison of STM images, nc-AFM images, and structures for molecular reactant and products. (A) STM image of **1** on Ag(100) before annealing. (B to D) STM images of individual products **2**, **3**, and **4** on Ag(100) after annealing at $T > 90^\circ\text{C}$ ($I = 10$ pA, $V = -0.2$ V, $T = 4$ K). (E) nc-AFM image of the same molecule (reactant **1**) depicted in (A). (F to H) nc-AFM images of the same molecules (products **2**, **3**, and **4**) depicted in (B) to (D). nc-AFM images were obtained at sample bias $V = -0.2$ V (qPlus sensor resonance frequency = 29.73 kHz, nominal spring constant = 1800 N/m, Q-value = 90,000, oscillation amplitude = 60 pm). (I to L) Schematic representation of the molecular structure of reactant **1** and products **2**, **3**, and **4**. All images were acquired with a CO-modified tip.



provides insight into the detailed thermal reaction mechanisms that convert **1** into the products. We limit our discussion to the reaction pathways leading from **1** to the two most abundant products, **2** and **3**. The reactivity of *oligo*-1,2-diethynylbenzene **1** can be rationalized by treating the 1,2-diethynylbenzene subunits as independent but overlapping enediyne systems that are either substituted by two phenyl rings for the central enediyne, or by one phenyl ring and one hydrogen atom in the terminal segments. This treatment suggests three potential cyclizations along the reaction pathway (resulting in six-, five-, or four-membered rings) (22), in addition to other possible isomerization processes such as [1,2]-radical shifts or bond rotations that have been observed in related systems (23). Combinations of these processes leading to the products in a minimal number of steps were explored and analyzed using DFT calculations (14).

We started by calculating the total energy of a single adsorbed molecule of the reactant **1** on a Ag(100) surface. Activation barriers and the energy of metastable intermediates were calculated (including molecule-surface interactions) for a variety of isomeric structures along the reaction pathway leading toward the products **2** and **3**. Our observations that the structure of reactants on Ag(100) remains unchanged for $T < 90^\circ\text{C}$ and that no reaction intermediates can be detected among the products indicate that the initial enediyne cyclization is associated with a notable activation barrier that represents the rate-determining step in the reaction. In agreement with

experiments, DFT calculations predict an initial high barrier for the first cyclization reactions, followed by a series of lower barriers associated with subsequent bond rotations and hydrogen shifts.

Figure 3A shows the reaction pathway determined for the transformation of **1** into product **2**. The rate-determining activation barrier is associated with a $\text{C}^1\text{--C}^6$ Bergman cyclization of a terminal enediyne coupled with a $\text{C}^1\text{--C}^5$ cyclization of the internal enediyne segment to give the intermediate diradical **Int1** in an overall exothermic process (-60.8 kcal mol $^{-1}$). Rotation of the third enediyne subunit around a double bond, followed by the $\text{C}^1\text{--C}^5$ cyclization of the fulvene radical with the remaining triple bond, leads to **Int2**. The rotation around the exocyclic double bond is hindered by the Ag surface and requires the breaking of the bond between the unsaturated valence on the sp^2 carbon atom and the Ag. Yet the activation barrier associated with this process does not exceed the energy of the starting material used as a reference. The formation of three new carbon-carbon bonds and the extended aromatic conjugation stabilize **Int2** by -123.9 kcal mol $^{-1}$ relative to **1**. Finally, a sequence of radical [1,2]- and [1,3]-hydrogen shifts followed by a $\text{C}^1\text{--C}^6$ cyclization leads from **Int2** directly to the dibenzofulvalene **2**. Our calculations indicate that the substantial activation barriers generally associated with radical hydrogen shifts in the gas phase (50 to 60 kcal mol $^{-1}$) (24, 25) are lowered through the stabilizing effect of the Ag atoms on the surface, and thus they do not represent a rate-limiting process (Fig. 3A).

The reaction sequence toward **3** is illustrated in Fig. 3B. The rate-determining first step involves two $\text{C}^1\text{--C}^5$ cyclizations of the sterically less hindered terminal enediynes to yield benzo-fulvene diradicals. The radicals localized on the exocyclic double bonds subsequently recombine in a formal $\text{C}^1\text{--C}^4$ cyclization to yield the four-membered ring in the transient intermediate **tInt1**. This process involves the formation of three new carbon-carbon bonds, yet it lacks the aromatic stabilization associated with the formation of the naphthyl fragment in **Int2** and is consequently less exothermic (-60.7 kcal mol $^{-1}$). A sequence of bond rotations transforms **tInt1** via **Int3** into **tInt2**. Alignment of the unsaturated carbon valences in diradical **tInt1** with underlying Ag atoms maximizes the interaction with the substrate and induces a highly non-planar arrangement, thereby making subsequent rotations essentially barrierless. [1,2]-Hydrogen shifts and a formal $\text{C}^1\text{--C}^6$ cyclization yield the biphenylene **3**.

Both reaction pathways toward **2** and **3** involve $\text{C}^1\text{--C}^5$ enediyne cyclizations. These are generally energetically less favorable relative to the preferred $\text{C}^1\text{--C}^6$ Bergman cyclizations (22), but factors such as the steric congestion induced by substituents on the alkynes (12, 22), the presence of metal catalysts (26), or single-electron reductions of enediynes (27, 28) have been shown to sway the balance toward $\text{C}^1\text{--C}^5$ cyclizations yielding benzo-fulvene diradicals. All three of these factors apply to the present case of the thermally induced cyclization of **1** on Ag(100)

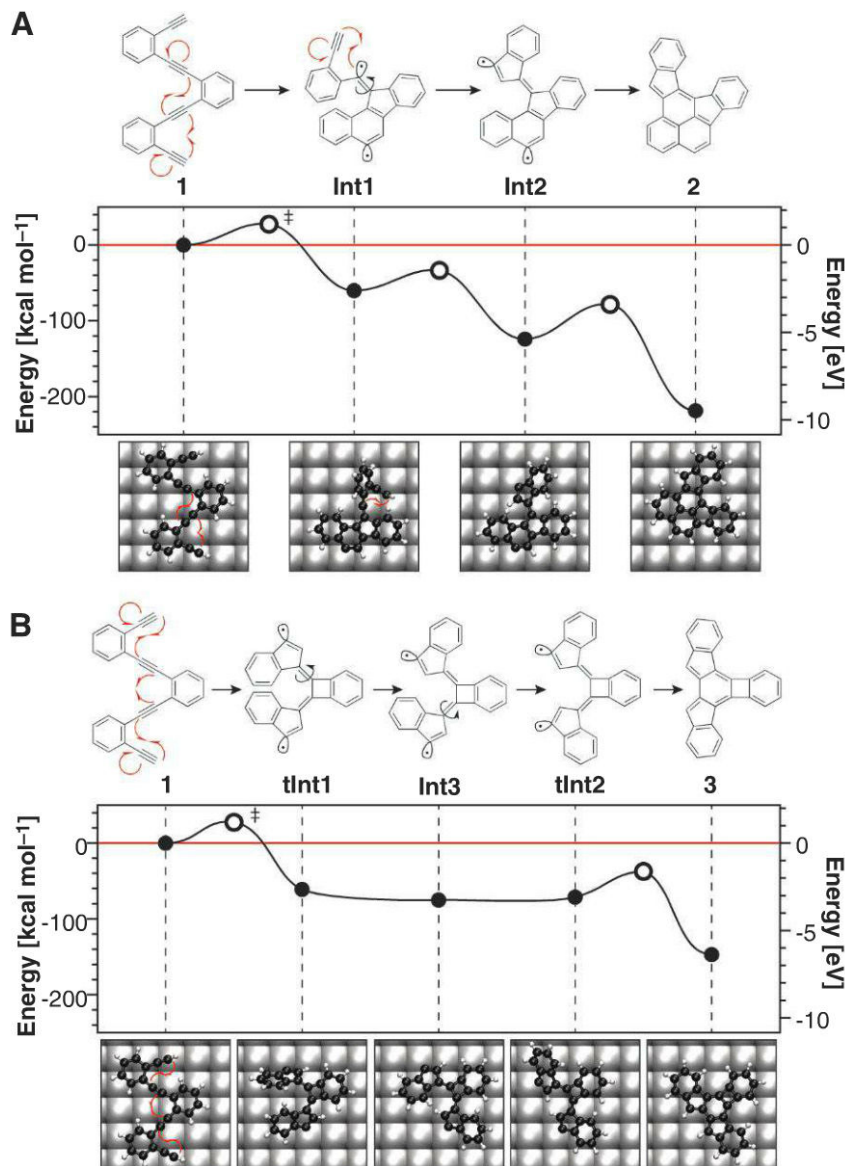


Fig. 3. Reaction pathways and their associated energies as calculated by DFT. (A) Proposed pathway for the cyclization of reactant **1** into product **2** on Ag(100). (B) Proposed pathway for the cyclization of reactant **1** into product **3** on Ag(100). Energies for **1**, **2**, and **3** (end point solid circles), for the intermediates **Int1**, **Int2**, **Int3**, **tlnt1**, and **tlnt2** (intermediate solid circles), and for the reaction barriers (open circles) were calculated using ab initio DFT theory (14). Ball-and-stick models show the nonplanar structure of intermediates. The symbol ‡ indicates the rate-determining transition state; the red line is the reference energy of **1** on Ag(100).

[e.g., bulky phenyl substituent on C¹ and C⁶, a metallic substrate, and a charge transfer of 0.5 electrons from the substrate to **1** (14)], thus facilitating the C¹–C⁵ cyclizations. The precise order of the low-energy processes (such as the **Int3**–**tlnt2** rotation and the **tlnt2**–**3** [1,2]-hydrogen shifts) following the rate-limiting initial cyclizations cannot be strictly determined experimentally. However, the sequence does not change the overall reaction kinetics and thermodynamics discussed above. Our bond-resolved single-molecule imaging thus allows us to extract an exhaustive picture and unparalleled insight into the chemistry involved in complex enediyne cyclization

cascades on Ag(100) surfaces. This detailed mechanistic understanding in turn guides the design of precursors for the rational synthesis of functional surface-supported molecular architectures.

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Supplementary Materials

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The Origins of Scaling in Cities

Luís M. A. Bettencourt

Despite the increasing importance of cities in human societies, our ability to understand them scientifically and manage them in practice has remained limited. The greatest difficulties to any scientific approach to cities have resulted from their many interdependent facets, as social, economic, infrastructural, and spatial complex systems that exist in similar but changing forms over a huge range of scales. Here, I show how all cities may evolve according to a small set of basic principles that operate locally. A theoretical framework was developed to predict the average social, spatial, and infrastructural properties of cities as a set of scaling relations that apply to all urban systems. Confirmation of these predictions was observed for thousands of cities worldwide, from many urban systems at different levels of development. Measures of urban efficiency, capturing the balance between socioeconomic outputs and infrastructural costs, were shown to be independent of city size and might be a useful means to evaluate urban planning strategies.

Cities exist, in recognizable but changing forms, over an enormous range of scales (1), from small towns with just a few people to the gigantic metropolis of Tokyo, with more than 35 million inhabitants. Many parallels have been suggested between cities and other complex systems, from river networks (2) and biological organisms (3–6) to insect colonies (1, 7) and ecosystems (8). The central flaw of all these arguments is their emphasis on analogies of

form rather than function, which limit their ability to help us understand and plan cities.

Recently, our increasing ability to collect and share data on many aspects of urban life has begun to supply us with better clues to the properties of cities, in terms of general statistical patterns of land use, urban infrastructure, and rates of socioeconomic activity (6, 9–13). These empirical observations have been summarized across several disciplines, from geography to economics, in terms of how different urban quantities (such as the area of roads or wages paid) depend on city size, usually measured by its population, N .

The evidence from many empirical studies over the past 40 years points to there being no special size to cities, so that most urban properties, Y , vary continuously with population size and are well described mathematically on average by power-law scaling relations of the form $Y = Y_0 N^\beta$, where Y_0 and β are constants in N . The surprise, perhaps, is that cities of different sizes do have very different properties. Specifically, one generally observes that rates of social quantities (such as wages or new inventions) increase per capita with city size (11, 12) (super-linear scaling, $\beta = 1 + \delta > 1$, with $\delta \approx 0.15$), whereas the volume occupied by urban infrastructure per capita (roads, cables, etc.) decreases (sublinear scaling, $\beta = 1 - \delta < 1$) (Fig. 1). Thus, these data summarize familiar expectations that larger cities are not only more expensive and congested, but also more exciting and creative when compared to small towns.

These empirical results also suggest that, despite their apparent complexity, cities may actually be quite simple: Their average global properties may be set by just a few key parameters (12, 13). However, the origin of these observed scaling relations and an explanation for the interdependencies between spatial, infrastructural, and social facets of the city have remained a mystery.

Here, I develop a unified and quantitative framework to understand, at a theoretical level, how cities operate and how these interdependencies arise. Consider first the simplest model of a city with circumscribing land area A and

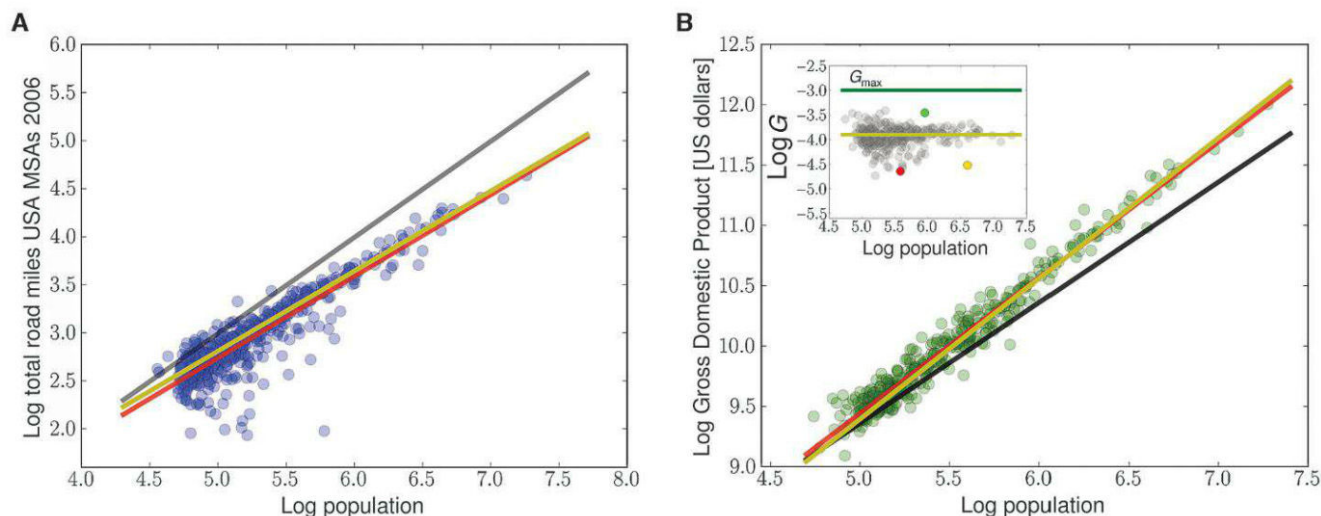


Fig. 1. Scaling of urban infrastructure and socioeconomic output. (A) Total lane miles (volume) of roads in U.S. metropolitan areas (MSAs) in 2006 (blue dots). Data for 415 urban areas were obtained from the Office of Highway Policy Information from the Federal Highway Administration (14). Lines show the best fit to a scaling relation $Y(N) = Y_0 N^\beta$ (red), with $\beta = 0.849 \pm 0.038$ [95% confidence interval (CI), $R^2 = 0.65$]; the theoretical prediction, $\beta = 5/6$ (yellow); and linear scaling $\beta = 1$ (black). **(B)** Gross metropolitan product of MSAs in 2006 (green dots). Data obtained for 363 MSAs from U.S. Bureau of Economic Analysis (14). Lines describe best fit (red) to data, $\beta = 1.126 \pm 0.023$ (95% CI, $R^2 = 0.96$); the theoretical prediction, $\beta = 7/6$ (yellow); and proportional scaling, $\beta = 1$ (black). The two best-fit parameters in each scaling

relation were estimated by means of ordinary least-squares minimization to the linear relation between logarithmically transformed variables (14). The inset shows the estimate of G for 313 U.S. MSAs and the conservation law $\frac{d \ln G}{d \ln N} = 0$ ($R^2 = 0.003$). G is measured as the product of gross domestic product and road volume, both per capita. As predicted by the theory, observed values of G for different cities cluster around its most likely value (mode, yellow line), which gives an estimate of the optimum G^* , and are bounded by the maximum $G_{max} \approx 8G^*$ (green line); see also Fig. 2B. Several metropolitan areas, such as Bridgeport, Connecticut (green circle); Riverside, California (yellow circle); or Brownsville, Texas (red circle), are outliers, suggesting that they are suboptimal in terms of their transportation efficiency or amount of social mixing.

population N . I write the interactions between people i, j in terms of a social network, F_{ij}^k , and assume that social interactions [e.g., friendship, employment, acquaintance, etc.] are local, take place over an interaction area a_0 (a cross section in the language of physics), and have strength g_k , where k describes social link types (14). The parameters, g_k , can be either positive (attractive, expressing a social benefit, e.g., mutually beneficial economic relations) or negative (repulsive, expressing a social cost, e.g., crime). All these processes share the same average underlying dynamics of social encounters in space and time, against the background of the city and its infrastructure networks.

The average number of local interactions per person is given by the product of the volume spanned by their movement, $a_0\ell$, times the population density $n = N/A$, where ℓ is the typical length traveled by people, goods, and information (14). The total average social output of a city can be obtained by multiplying the total number of interactions by the average outcome per interaction, $\bar{g}$, leading to $Y = G \frac{N^2}{A}$, with the parameter $G \equiv \bar{g}a_0\ell$ measuring the product of average social output times area, both per capita (Fig. 1). Each urban socioeconomic output, Y , has physical units set by g_k , but it is useful to think of all quantities ultimately expressed in terms of energy per unit time (power).

Another crucial property of cities is that they are mixing populations. That is, even if people in the city explore different locations at different times, anyone can in principle be reached by anyone else. This concept, familiar from population biology (15), is the basis of definitions of functional cities as metropolitan statistical areas (MSAs), e.g., by the U.S. census bureau. In practice, this means that the cost per person of a mixing population is proportional to the transverse dimension (diameter), L , of the city $L \sim A^{1/2}$. Thus, the total power spent in transport processes to keep the city mixed is $T = \epsilon LN = \epsilon A^{1/2}N$, where ϵ is a force per unit time. This cost must be covered by each individual's budget, $y = Y/N$, requiring $y \approx T/N$, which implies $A(N) = aN^\alpha$ with $\alpha = 2/3$ and $a = (G/\epsilon)^\alpha$. The baseline area, a , increases with more productive interactions, e.g., due to economic growth, and decreasing transportation costs, as is observed in worldwide patterns of urban sprawl over time (16). Thus, I obtain $Y = Y_0N^\beta$, where $\beta = 2 - \alpha = 1 + 1/3 > 1$ and $Y_0 = G^{1-\alpha}\epsilon^\alpha$. This simple model leads to area, A , varying sublinearly with N ($\alpha = 2/3 < 1$), and socioeconomic outputs, Y , varying superlinearly ($\beta = 4/3 > 1$). However, this overestimates β because as cities grow, space becomes occupied and transportation of people, goods, and information is channeled into networks. The space created by these networks gives the correct measure of the social interactions that can occur in cities.

I propose a more realistic model by generalizing these ideas in terms of four simple assumptions:

1) Mixing population. The city develops so that citizens can explore it fully given the re-

sources at their disposal. I formalize this principle as an entry condition (17), by requiring that the minimum resources accessible to each urbanite, $Y_{\min}/N \sim GN/A$, match the cost of reaching anywhere in the city. Because travel paths need not be linear, I generalize their geometry via a fractal dimension, H , so that distance travelled $\propto A^{H/D}$ (14). Matching interaction density to costs, I obtain a generalized area scaling relation, $A(N) = aN^\alpha$, with a as before and $\alpha = \frac{2}{2+H}$ [$\alpha = \frac{D}{D+H}$ in D dimensions]. $H = 1$ allows individuals to fully explore the city within the smallest distance traveled, implying that N scales like a physical volume (14, 18).

2) Incremental network growth. This assumption requires that infrastructure networks develop gradually to connect people as they join, leading to decentralized networks (6, 19). Specifically, the scaling of Fig. 1A is obtained when the average distance between individuals $d = n^{-1/2} = (A/N)^{1/2}$ equals the average length of infrastructure network per capita so that the total network area, $A_n(N) \sim Nd = A^{1/2}N^{1/2}$. Together with the first assumption, this implies that $A_n \sim a^{1/2}N^{1-\delta}$ with $\delta = 1/6$ [$A_n \sim A^{1/D}N^{(D-1)/D} = a^{1/D}N^{1-\delta}$, with $\delta = \frac{H}{D(D+H)}$ in D dimensions]. This has been observed in U.S. and German road networks (6, 12, 19) and tracks the average built area of more than 3600 large cities worldwide (16), measured through remote sensing.

3) Human effort is bounded, which requires that G is, on average, independent of N , i.e., $dG/dN = 0$ (Fig. 1B, inset). The increasing mental and physical effort that growing cities can demand from their inhabitants has been a pervasive concern to social scientists (20). Thus, this assumption is necessary to lift an important objection to any conceptualization of cities as scale-invariant systems. Bounded effort is also observed in urban cell phone communication

networks (21) and is in general a function of human constraints and urban services and structure.

4) Socioeconomic outputs are proportional to local social interactions, so that $Y = GN^2/A_n \sim N^{1+\delta}$. From this perspective, cities are concentrations not just of people, but rather of social interactions. This point was emphasized by Jacobs (22, 23), but has been difficult to quantify. The prediction that social interactions scale with $\beta = 1 + \delta \approx 7/6$ was observed recently in urban telecommunication networks (21). Together these assumptions predict scaling exponents for a wide variety of urban indicators, from patterns of human behavior and properties of infrastructure to the price of land (6, 9–12, 16, 21, 24, 25), summarized in Table 1 (14).

Thus far, I obtained estimates for scaling exponents without the need for a detailed model of infrastructure. Next, I show how network models of infrastructure can help to illuminate urban planning issues. Consider the infrastructure in a city described by a network with h hierarchical levels (Fig. 2A). The network branching, b , measures the average ratio of the number of units of infrastructure at successive levels, $N_i = b^i$, e.g., number of paths to small roads, or larger roads to highways. I assume that the number of infrastructure units at the lowest level, $i = h$, equals the number of people, so that $N_h = N$ and $h = \ln N / \ln b$. These networks are not hierarchical trees (26) (Fig. 2A). The length of a network segment (such as a road) at level i is l_i , crossing a land area a_i , and its transverse dimension is s_i , an area in 3D networks and a length in 2D. To obtain the above scaling relations, I assume that the transverse dimension of the smallest network units, s_* , is independent of N . This leads to the scaling of network width, $s_i = s_* b^{(1-\delta)(h-i)}$, which says that highways or water mains are much wider than building corridors or household

Table 1. Urban indicators and their scaling relations. Columns show measured exponent ranges (see table S3 for details). Also shown are predicted values for $D = 2$, $H = 1$ (the simplest theoretical expectation) and for general D, H . Agglomeration effects vanish as $H \rightarrow 0$ (14). The larger range for the observed land-area exponent is likely the result of different definitions of the city in space and distinct measurement types. See table S3 and supplementary text for specific values of observed exponents, discussion, and additional data sources.

Urban scaling relations	Observed exponent range	Model ($D = 2, H = 1$)	Model D, H
Land area $A = aN^\alpha$	[0.56,1.04]	$\alpha = \frac{2}{3}$	$\alpha = \frac{D}{D+H}$
Network volume $A_n = A_0N^\nu$	[0.74,0.92]	$\nu = \frac{5}{6}$	$\nu = 1 - \delta$
Network length $L_n = L_0N^\lambda$	[0.55,0.78]	$\lambda = \frac{2}{3}$	$\lambda = \alpha$
Interactions per capita $\bar{l}_i = l_0N^\delta$	[0.00,0.25]	$\delta = \frac{1}{6}$	$\delta = \frac{H}{D(D+H)}$
Socioeconomic rates $Y = Y_0N^\beta$	[1.01,1.33]	$\beta = \frac{7}{6}$	$\beta = 1 + \delta$
Network power dissipation $W = W_0N^\omega$	[1.05,1.17]	$\omega = \frac{7}{6}$	$\omega = 1 + \delta$
Average land rents $P_L = P_0N^{\delta_L}$	[0.46,0.52]	$\delta_L = \frac{1}{2}$	$\delta_L = 1 - \alpha + \delta$

pipes, $s_0 = s_* b^{(1-\delta)h} \gg s_h = s_*$. Additionally, because infrastructure must reach everyone in the city (6, 18), total network length is area filling, $l_i = a_i/l$, with $a_i = ab^{(\alpha-1)i}$. This means that the land area per person, $a_h = aN^{\alpha-1}$, and shortest network distance, $l_h = (a/l)N^{\alpha-1}$, which defines l , decrease with N . The total network length L_n and network area A_n follow from the sum of the geometric series over levels

$$L_n = \sum_{i=0}^h l_i N_i = \frac{a}{l} \sum_{i=0}^h b^{ai} = \frac{a b^{\alpha(h+1)} - 1}{l(b^\alpha - 1)} \approx L_0 N^\alpha, L_0 = a/l \quad (1)$$

$$A_n = \sum_{i=0}^h s_i l_i N_i = s_* \frac{a}{l} b^{(1-\delta)h} \sum_{i=0}^h b^{(\alpha+\delta-1)i} \approx A_0 N^{1-\delta}, A_0 = \frac{s_* a}{l(1-b^{\alpha+\delta-1})} \quad (2)$$

where I took $\alpha + \delta < 1$, which holds for $D > 1$.

I can now compute the cost of maintaining the city connected as the energy necessary for moving people, goods, and information across its infrastructure networks. These movements form a set of currents, transporting various quantities across the city and can be quantified by means of the language of circuits. The scaling of s_i together with total current, J , conservation across levels $J_i = s_i \rho_i v_i N_i = s_{i-1} \rho_{i-1} v_{i-1} N_{i-1} = J_{i-1}$ for all i , sets the scaling for $\rho_i v_i$, the current density at level i , where ρ_i is the density of carriers in the network and v_i their average velocity. This quantity is interesting because it controls the dissipation mechanisms in any network. I obtain $\rho_i v_i = b^{-\delta} \rho_{i-1} v_{i-1}$, which implies that the current density decreases with increasing i , so that highways are faster and/or more densely packed than smaller roads (27, 28). Making the additional assumption that individual needs, $\rho_h v_h = \rho_* v_*$, are independent of N (12) leads to $\rho_i v_i = b^{\delta(h-i)} \rho_* v_*$. Then, the total current $J_i = J = J_0 N$, with $J_0 = s_* \rho_* v_*$, which is a function only of individuals' characteristics.

There are many forms of energy dissipation in networks, including those that occur at large velocity or density. Here, I make the standard assumption that the resistance per unit length per transverse network area, r , is constant (2, 5), leading to the resistance per network segment, $r_i = r \frac{l_i}{s_i}$. For N_i parallel resistors this gives the total resistance per level, $R_i = \frac{r_i}{N_i} = \frac{ar}{ls_*} b^{-(1-\alpha+\delta)i-(1-\delta)h}$. The total power dissipated, W , follows from summing $W_i = R_i J_i^2$ over levels,

$$W = J^2 \sum_{i=1}^h R_i = J^2 \frac{ar}{ls_*} b^{-(1-\delta)h} \frac{1-b^{-(1-\alpha+\delta)(h+1)}}{1-b^{-1+\alpha-\delta}} \approx W_0 N^{1+\delta}, W_0 = \frac{arJ_0^2}{ls_* (1-b^{-1+\alpha-\delta})} \quad (3)$$

which scales superlinearly, with exponent $1 + \delta = 1 + 1/6$ in $D = 2$, $H = 1$. Thus, energy dissipation scales with population like social interactions, as observed in German urban power grids (12), so that the ratio Y/W , a measure of urban efficiency, is independent of city size.

Finally, I show that these results can be derived by maximizing net urban output, $\mathcal{L}$, as the difference between social interaction outcomes, Y , and infrastructure energy dissipation, W , under settlement and network constraints,

$$\mathcal{L} = Y - W + \lambda_1 (\epsilon A^{H/D} - GN/A) + \lambda_2 (A_n - cNd) \xrightarrow{d\mathcal{L}/dG=0} \frac{2\alpha-1}{\alpha} G^* \frac{N^2}{A_n(N)} \quad (4)$$

where $c = A_0 a^{-1/D}$ and λ_1, λ_2 are Lagrange multipliers. Equation 4 gives the basis for the derivation of the properties of every segment in the network, through Eqs. 1 and 2, in analogy with (2, 4, 5). The novelty in Eq. 4 is the prediction of an optimal $G = G^*$, through $d\mathcal{L}/dG = 0$, and the expectation that values of G for different cities

fluctuate around this value, as observed in Fig. 1B (inset).

To see this, consider that, keeping ϵ fixed and $a = (G/\epsilon)^\alpha$, both Y and W grow with G , because $Y_0 \sim G^{1-\alpha}$ and $W_0 \sim G^\alpha$. This tension between social interactivity, transportation costs, and spatial settlement patterns is at the root of most urban planning and policy. The limiting values of G follow from the solutions to $\mathcal{L} = 0$: $G = 0$ and

$$G = G_{\max} = \left[\frac{(\epsilon l)^{2\alpha}}{r' J_0^2} l^{2(1-\alpha)} \right]^{\frac{1}{2\alpha-1}}, \text{ where } r' \approx r \text{ (14). It}$$

follows that $G^* = \left(\frac{1-\alpha}{\alpha} \right)^{1/(2\alpha-1)} G_{\max} \approx G_{\max}/8$, with $\alpha \approx 2/3$ (Fig. 1B, inset). Thus, cities will form if the balance of social interactions is positive, $\bar{g} > 0$. However, there is an upper value of $G = G_{\max}$ (Fig. 1B, inset) beyond which dissipation costs overcome social benefits and a city may split up into regions. For $G < G^*$, the social interaction potential of a city is underdeveloped. Such places tend to be poorer and have less advanced infrastructure. Thus, I would expect that cities such as Riverside, California, or Brownsville, Texas (Fig. 1B), where estimates of G are less than average, would typically benefit from measures

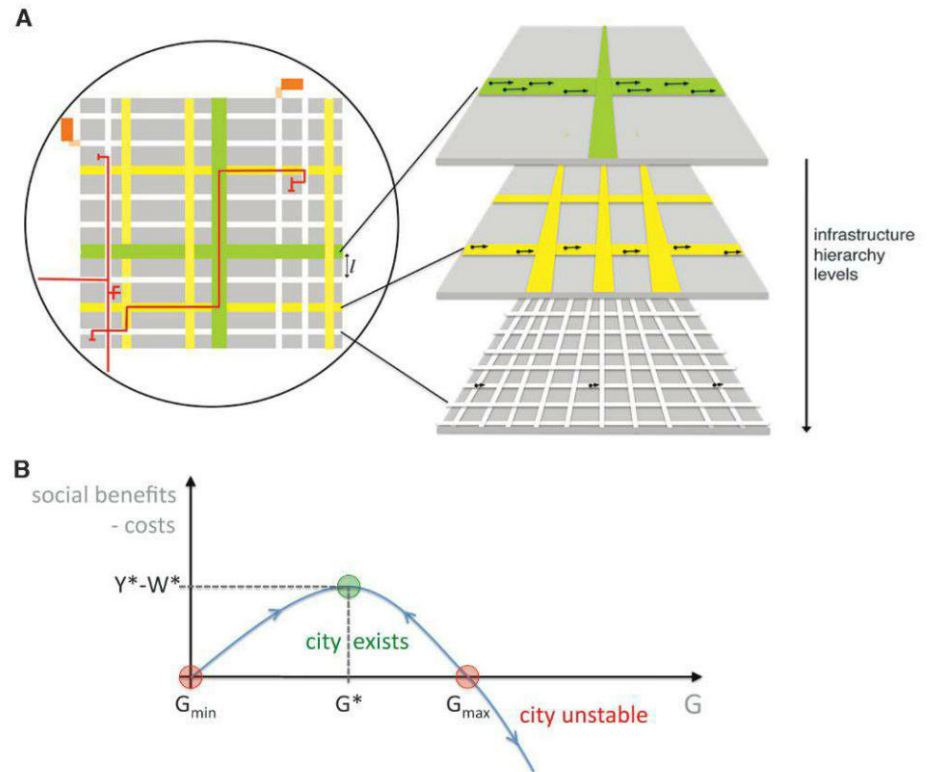


Fig. 2. The spatial city and its social and dissipative processes. (A) Gray blocks denote settled areas, and spaces in between (white, yellow, green) represent infrastructure networks, treated in terms of a size hierarchy. Total network length $L_n = 2(n_b + 1)L \approx A/l$ is area filling (circle), where n_b is the number of blocks across the city (14). Red lines denote the volume of public space spanned by an individual, which determines his or her average number of social interactions. As the city grows and new land is settled (orange blocks), the infrastructure network grows incrementally (orange segments). The flux $\rho_i v_i$ in larger network segments is higher (black dots plus arrows), controlling the energy dissipation in the city. (B) There is an optimal value of G at which cities are most productive. Cities can exist when social interactions are positive $G > G_{\min} = 0$, and less than an upper value $G < G_{\max}$ (red circles), at which point dissipation costs overcome benefits. The optimal $G = G^*$ (green circle) corresponds to the most efficient city.

that promote greater mobility or density, in order to achieve more intense and beneficial city-wide social contact. Conversely, cities with $G > G^*$ become victims of their socioeconomic success by incurring escalating mobility costs. Bridgeport, Connecticut's, MSA (Fig. 1B) may be developed in terms of its economic functions and infrastructure, but might generally benefit from more compact urban living or from increases in transportation energy efficiency. That is, cities may be suboptimal either because they do not realize their full social potential or because they do so in a manner that renders transportation costs too high. In either case, this approach shows how urban planning must take into account the delicate net balance between density, mobility, and social connectivity and thus provides a general framework for the iterative development and assessment of urban policies.

That many cities are becoming more global in their economic relations and political and cultural influence (29) does not alter the basic premises of the theory. The internal dynamics and organization of cities (as social networks of people and institutions) produces new socioeconomic functions that allow cities to exchange goods, services, people, and information within and across national borders (22, 23, 30). Thus, even if some singular places such as Hong Kong, Singapore, or Dubai are primarily part of international economies, the majority of the world's most global cities, such as Tokyo, New York, Los Angeles, Beijing, Shanghai, Berlin, or Frankfurt, show clear scaling effects in line with their own national urban systems (Fig. 1 and figs. S1 to S3).

All cities have spatial and social pockets of greater and lower mobility, social integration, better or worse services, and so forth (1, 17). It should be emphasized that the theory does not predict density profiles or socioeconomic differences inside the city, but the scaling for the properties of the city as a whole. None of these pockets exist in absolute isolation; they are just more or less "connected," so they must be understood with reference to the rest of the city (17).

The interactions between people also provide the basis for institutional relationships via the appropriate groupings of individuals in social or economic organizations and by the consideration of the resulting links between such entities. Institutions and industries that benefit from strong mutual interactions may aggregate in space and time within the city in order to maximize their $Y - W$, a point first made by Marshall (23) in the context of industrial districts. Other organizations may benefit primarily from the general effects that result from being in the wider city and collecting a diversity of interactions, an argument often attributed to Jacobs (22). These results establish necessary conditions for urban areas to express certain levels of socioeconomic productivity, but it remains a statistical question (21, 25) how well they are realized in specific places.

Most urban systems for which reliable data exist confirm almost exactly the simplest predictions of the theory developed here. Examples

are the scaling of area for about 1800 cities in Sweden (14, 18), or for roads in several hundred American (Fig. 1A) and Japanese metropolitan areas (fig. S3). One of the most spectacular agreements is for the scaling of total area of paved surfaces for all cities worldwide above 100,000 people (over 3600 cities) (14, 16). These examples illustrate the result derived above that urban infrastructure volume scales faster with population than land area (and both are sublinear). This effect is visually apparent in large, developed cities, where roads, cables, and pipes become ubiquitous and eventually migrate into the third dimension, above or below ground.

Measurements of electrical cable length and dissipative losses in German urban power grids (12) further confirm these expectations and support another key result obtained above: The energy loss in transport processes scales like socioeconomic rates (and both are superlinear). This shows how cities are fundamentally different from other complex systems, such as biological organisms (4, 5) or river networks (2), which are thought to have evolved to minimize energy dissipation. Thus, the framework developed here also brings into focus efforts for sustainable urban development, by showing what kind of energy budget must be expended in order to keep cities of varying sizes socially connected.

The predictions of the theory are further supported by data on the size of urban economies from hundreds of cities in several continents, such as those in the United States (Fig. 1B), Japan (fig. S3), China (fig. S2A), or Germany (fig. S2B). In particular, the specific result that scaling exponents remain invariant over time, and are independent of population size and level of development, is confirmed by data for wages in U.S. metropolitan areas spanning 40 years (fig. S3). Direct empirical tests on the predictions made here for individual properties remain more difficult, but are confirmed, for example, by measurements for the scaling of social interactions with city size in the cell phone networks of two European nations (21), and for certain other patterns of individual behavior (12, 20, 31). Nevertheless, for most nations, we cannot yet access all predicted urban quantities simultaneously, especially in developing countries. This provides many future tests and applications for the theory, especially where understanding urbanization is most critical.

The spatial concentration and temporal acceleration of social interactions in cities has some striking qualitative parallels in other systems that are also driven by attractive forces and become denser with scale (20, 30). The most familiar are stars, which burn faster and brighter (superlinearly) with increasing mass. Thus, although the form of cities may resemble the vasculature of river networks or biological organisms, their primary function is as open-ended social reactors. This view of cities as multiple interconnected networks that become denser with increasing scale (32) may also help to elucidate the function of other systems with similar properties, from ecosystems

to technological information networks, despite their different relationships to physical space.

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Supplementary Materials

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Topology of Feather Melanocyte Progenitor Niche Allows Complex Pigment Patterns to Emerge

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Color patterns of bird plumage affect animal behavior and speciation. Diverse patterns are present in different species and within the individual. Here, we study the cellular and molecular basis of feather pigment pattern formation. Melanocyte progenitors are distributed as a horizontal ring in the proximal follicle, sending melanocytes vertically up into the epithelial cylinder, which gradually emerges as feathers grow. Different pigment patterns form by modulating the presence, arrangement, or differentiation of melanocytes. A layer of peripheral pulp further regulates pigmentation via patterned agouti expression. Lifetime feather cyclic regeneration resets pigment patterns for physiological needs. Thus, the evolution of stem cell niche topology allows complex pigment patterning through combinatorial co-option of simple regulatory mechanisms.

The color of avian plumage can be patterned within feathers and along the body axes. Pigmentation adapts to seasonal changes and physiological needs (1–4). Complex pigment patterns have coevolved with feather shapes to generate spectacular plumage, as seen in male peacocks, which inspired Darwin to propose sexual selection (5). Fossil evidence shows that feathered

dinosaurs exhibited feather pigment patterns (6, 7). Compared with their reptile ancestors endowed with multiple types of chromatophores, birds have only one pigment cell type, melanocyte (8). Feathers and hairs are ectodermal organs derived from convergent evolution (4, 9), and melanocytes in both display color variation and share basic molecular pathways (4, 8). In hair, melano-

cyte stem cells (McSCs) reside in the follicle bulge (10). Upon activation, they give rise to progeny in the matrix to color hair fibers (10, 11). Feather colors are complex (fig. S1), and the mechanisms for pigment patterning are poorly understood (2, 12). Here, we explore the cellular and molecular basis of pigment pattern formation.

First, we mapped McSCs in regenerating feather follicles. In the growing phase, melanocytes (pigmented or apigmented) were found in the epithelium above papilla ectoderm (Fig. 1, A and

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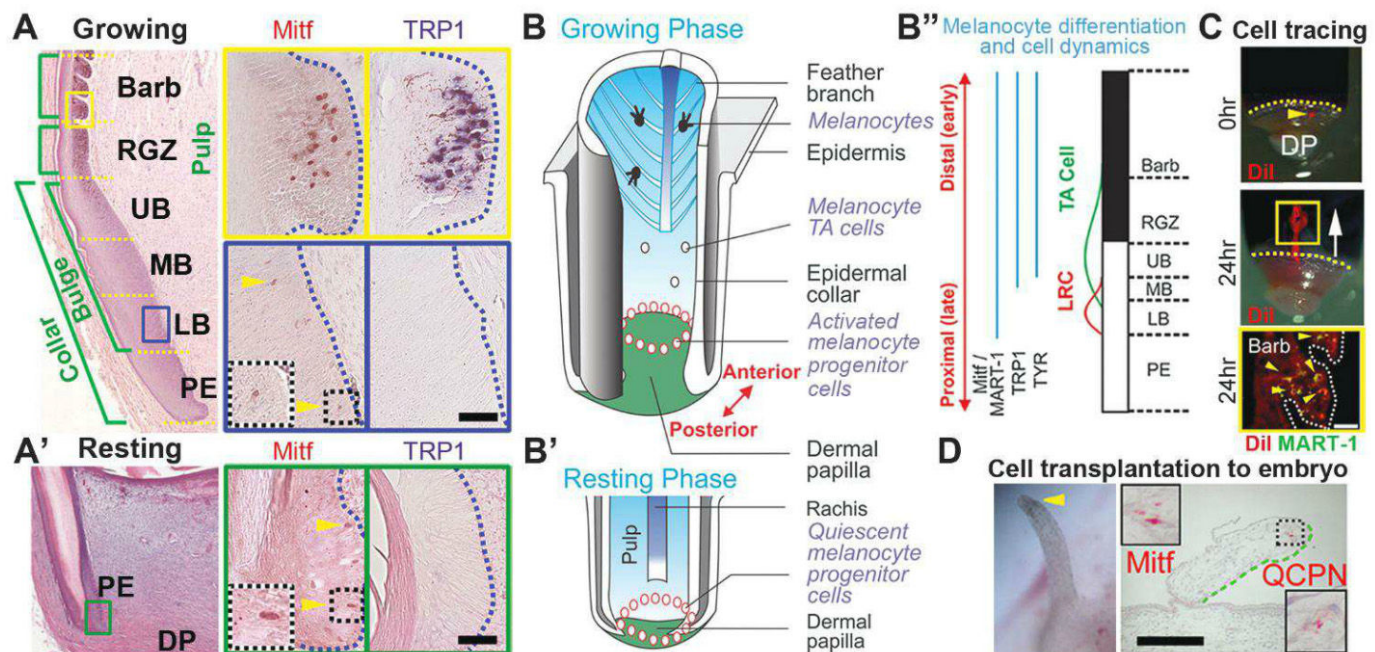


Fig. 1. Identification of feather melanocyte progenitor and its niche. (A) In the growing phase, undifferentiated melanocytes were in the lower bulge (blue box) and differentiated melanocytes in the barbs (yellow box). (A') In the resting phase, undifferentiated melanocytes were in the papilla ectoderm (green box). Progenitors are indicated by yellow arrowheads. (B and B') Schematic drawings showing feather follicle and cells within. (B'') Summary of melanocyte progenitor markers (fig. S2). (C) Dil labeling at LB (yellow arrowhead, 0 hour) and tracing showed that labeled cells moved upward to barb ridges (yellow box, 24 hours; white arrow indicates direction of

cell movement). Some Dil-positive cells expressed melanocyte marker MART-1 in the barb ridges at 24 hours of tracing (yellow arrowheads, lower panel). (D) Cell transplantation from quail resting papilla ectoderm (from black feather) to White Leghorn chicken embryo. The developing feather bud became pigmented (yellow arrowhead, left). Quail cell-specific perinuclear antigen (QCPN) staining confirmed that Mitf⁺ pigment cells were derived from quails (right). LB, lower bulge; LRC, label-retaining cell; MB, middle bulge; PE, papilla ectoderm; RGZ, ramosogenic zone; TA, transit amplifying; UB, upper bulge. Scale bars: 50 μ m in (A), (A'), and (C); 150 μ m in (D).

B, and fig. S2). Among different chicken breeds and non-Gallus birds, we found small apigmented $Mitf^+$ MART-1 $^+$ melanocytes in the lower to middle collar bulge region (Fig. 1B and fig. S3). Melanocytes in the lower bulge were negative for differentiation markers TRP1 and TYR (Fig. 1A and fig. S2B) and thus represented melanocyte progenitors. From the middle bulge and above, there were progressively increasing levels of $Mitf$, MART-1, TRP1, TYR, and melanin, as well as increases in melanocyte cell size and dendricity (Fig. 1, B and B', and fig. S2B).

To gauge their cell proliferative behavior, we employed bromodeoxyuridine (BrdU) labeling. Immature melanocytes in the lower bulge retained the label longer than those in the middle bulge or above (fig. S4A). Lower-bulge melanocytes were of low density (fig. S5) and exhibited low levels of proliferating cell nuclear antigen (PCNA) ($17.4 \pm 6.5\%$). These slow-cycling apigmented melanocytes retained BrdU for 8 days, much shorter than hair McSCs that retain label for about 70 days (10). The results indicate that melanocyte progenitors cycle more actively in feathers than

in hairs. To track the movement of their progeny, diiodoacetyl-tetramethylindocarbocyanine perchlorate (DiI) labeling revealed a vertically upward melanocyte flow from the lower bulge to the barb ridges (Fig. 1C). In vitro experiments also showed that these cells could proliferate and differentiate (fig. S4B). Hence, the immature melanocytes in the lower bulge are melanocyte progenitors, possibly including McSCs.

We then examined dynamic changes during feather cycling (9). When feathers entered the resting phase, melanocyte progenitors descended to the papilla ectoderm and became negative for PCNA (Fig. 1, A' and B', and fig. S6), consistent with a quiescent state. Transplantation of melanocyte progenitors from adult quail pigmented feathers to chicken embryos showed that quail cells can be incorporated into developing feather buds and produce pigment (Fig. 1D). As feather follicle epithelium is cylindrical in configuration, melanocyte progenitors are distributed in a ring in three dimensions with distinctive dynamic behavior in different feather cycle phases (Fig. 1, B and B', and figs. S3C and S6C).

To explore pigment patterning, we first determined whether feather whiteness is achieved by one or multiple cellular mechanisms. White feathers from White Leghorn, Speckled Sussex, and Delaware chickens were examined. Apigmentation in barbs could be caused either by an absence of melanocytes or by suppressed melanocyte differentiation (figs. S7 and S15A). When melanocytes were absent from barb ridges, progenitors could be either present or absent in the stem cell niche (figs. S7 and S15A). Thus, one color pattern can be achieved using different cellular mechanisms.

Feathers are made from the distal to proximal end, so we explored the roles of temporal control in pigment patterning. Flight feathers from Taiwan Country chickens were studied because the feather changed from a distal black vane to a proximal white calamus (fig. S8A), suggesting a temporal transition of melanocyte behavior. When the apigmented calamus was forming, melanocytes were still present in the lower collar. Only limited numbers of pigmented melanocytes were found in the middle collar (0 to 3 cells per section). Although keratinocytes still proliferated to form the calamus, melanocyte progenitors were negative for PCNA (fig. S8B). Hence, pigment patterns can form by temporal regulation of melanocyte progenitor activity. Some progenitors are preserved for the next regenerative cycle, and patterning potential is conserved.

We then explored how periodically striped patterns can be created. The horizontal barring of Barred Plymouth Rock feathers (Fig. 2A) is a classical model for pigment patterning (13, 14). This fascinating sex-linked trait is semidominant; homozygous males have wider white bands than heterozygous males, and previous work has implicated both coding and noncoding variation at the *CDKN2A/B* tumor suppressor locus as a

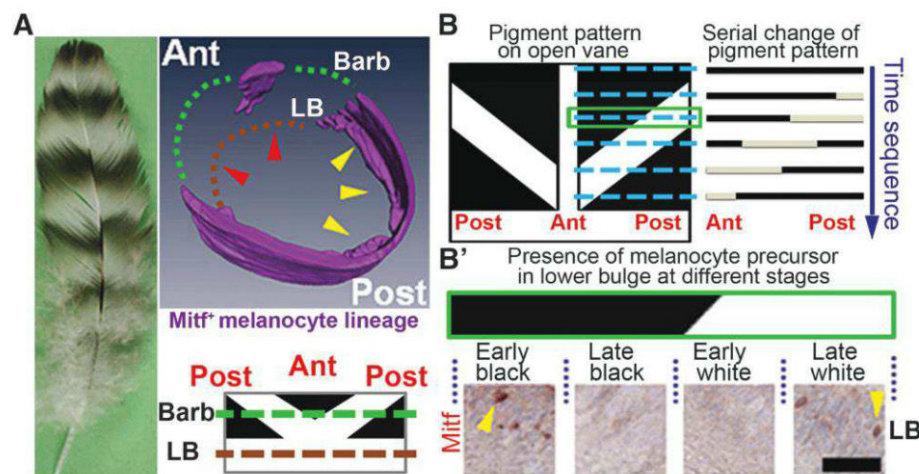


Fig. 2. Melanocyte progenitor niche topology allows periodic stripe formation in feathers from Barred Plymouth Rock chickens. (A) Three-dimensional reconstruction of $Mitf^+$ melanocyte lineage (purple) showed presence/absence (yellow/red arrowheads, respectively) of progenitors in LB. Lower panel depicts the pigment pattern when the specimen was sampled. Green/brown dashed lines indicate the level of barbs/LB, respectively. (B) Pigment patterns formed at different time points showed a shift of alternating black and white bands toward the anterior. (B') The boxed pigment pattern in (B) was divided into four phases. Melanocyte progenitors were present only in late white and early black (yellow arrowheads). Ant, anterior; Post, posterior. Scale bar: 50 μ m.

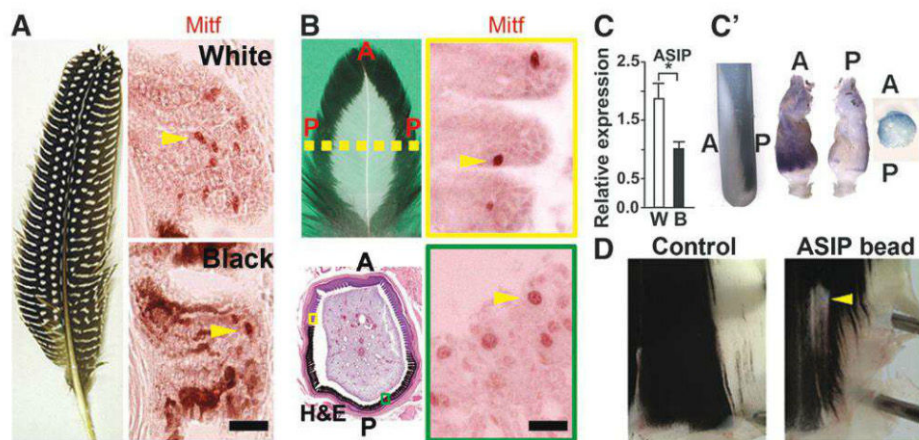


Fig. 3. Peripheral pulp inhibits melanocyte differentiation. (A) Feather from guineafowl. $Mitf^+$ melanocytes were present in both pigmented and apigmented barb ridges (yellow arrowheads). (B) Breast feather from Silver Laced Wyandotte chicken. The dashed line indicates the sampling time point. $Mitf^+$ melanocytes (yellow arrowheads) were present in both white and black barb ridges. (C) qPCR showed higher ASIP expression in the white region. * $P < 0.05$ ($N = 3$). (C') Whole-mount in situ hybridization demonstrated higher ASIP expression in peripheral pulp adjacent to apigmented epithelium. (D) Insertion of ASIP-coated beads into growing follicles in vivo induced a white stripe (yellow arrowhead). A, anterior; P, posterior; B, black; W, white. Scale bar: 25 μ m.

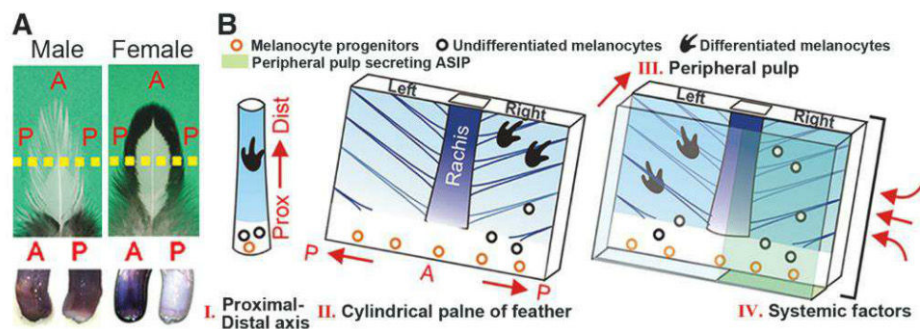


Fig. 4. Physiological regulation and principles of feather pigment pattern formation. (A) Wing covert feathers in adult Silver Laced Wyandotte chickens showed sexually dimorphic pigment patterns (upper panel). Whole-mount in situ hybridization revealed higher ASIP expression corresponding to apigmented regions (lower panel). Dashed line indicates the sampling time. (B) Multidimensional regulation of simple melanocyte behavior (fig. S15) allows temporospatial regulatory freedom. A, anterior; P, posterior.

potential cause (14). When the growing feather follicle cylinder was cut open vertically from the posterior side, a chevron-shaped barring pattern was revealed (fig. S9). Serial sections showed that the bars traveled toward the anterior side (Fig. 2B). The melanocyte lineage was absent in and below the apigmented regions (Fig. 2A), but melanocyte progenitors were still preserved in posterior collar bulge. Thus, black and white barbs can be divided into four phases (early black, late black, early white, and late white) with a fluctuating presence of melanocyte progenitors (Fig. 2B' and fig. S10, A to B'). Loss of melanocyte progenitors is caused by premature differentiation, not apoptosis (fig. S10C). The cyclic presence and absence of melanocyte progenitors implies a feedback mechanism (figs. S10D and S11). The results are consistent with a gain-of-function alteration in the CDKN2A/B tumor suppressor locus that causes premature melanocyte progenitor differentiation in a temporally and topologically restricted manner. The genetic defect in Barred Plymouth Rock chickens may cause aberrant feedback control that displays as repetitive stripes (14) (fig. S11). Chevron patterning and the circular McSC niche topology make the periodicity possible by allowing deletion of progenitors in the anterior while preserving progenitors in the posterior niche. There are certainly other striped feathers that are not based on this genetic defect (3), but we expect them to follow similar principles (figs. S11 and S15B).

The two-dimensional feather vane allows more complex pigment patterns to form. Feathers from guineafowls, Silver Spangled Hamburg, and Silver Laced Wyandotte chickens show multiple spots, a single distal spot, and a laced edge, respectively (fig. S12). Melanocytes were present in apigmented regions but remained undifferentiated (Fig. 3, A and B, and figs. S12 and S13, A to D). To test whether these cells could still differentiate, melanocytes from apigmented barbs were cultured. Under α -melanocyte-stimulating hormone (α -MSH) stimulation, they were induced to express tyrosinase activity (fig. S13, E and F).

Unexpectedly, this rescue failed when they were cocultured with adjacent pulp (fig. S13G), suggesting that pigment modulators might be secreted from adjacent mesenchyme. Agouti signaling protein (ASIP), an antagonist of α -MSH (15, 16), is an important mammalian pigment modifier capable of switching eumelanogenesis to pheomelanogenesis and regulating melanocyte precursor behavior in hair follicles (17–20). Its role in feather pigmentation has been proposed but not well defined (21). We searched for sources of pigment modulators by quantitative polymerase chain reaction (qPCR) and whole-mount in situ hybridization and found that ASIP was consistently up-regulated in peripheral pulp adjacent to apigmented barbs (Fig. 3, C and C', and fig. S13H). When we inserted ASIP-coated beads into the pulp adjacent to regenerating black feather barbs, a white zone was produced from the distal toward the proximal, reflecting inhibition of melanocyte differentiation during feather growth (Fig. 3D and fig. S13I). Thus, activators and inhibitors can work together to modulate pigment pathways and produce a spectrum of complex patterns (22–24).

A unique feature of plumage regeneration is that feathers can change from a downy to sexually dimorphic appearance for physiological needs (1) (fig. S14A). Feathers with different pigment patterns grew from the same follicle and had similar melanocyte precursor distributions (fig. S14B). Altered ASIP expression in the pulp of sexually dimorphic feathers corresponded to apigmented regions with suppressed melanocyte differentiation (Fig. 4A). Classical transplantation experiments that swapped feather germs between different follicles suggested that dermal papillae with attached papilla ectoderm serve as the signaling center that stores color patterning information (25). Thus, properties of the stem cell niche can change in response to systemic factors, and this effect can be mediated by mechanisms including altered ASIP expression in the pulp. Although inhibition of melanocyte differentiation by the mesenchyme occurs in human palmoplantar skin (26), the pattern is fixed for life. In birds, an

additional dimension of control has evolved to pattern ASIP expression in the pulp dynamically in different regenerating cycles, permitting melanocyte progenitor activity to be modulated by systemic hormones.

In summary, we found several principles behind the formation of complex pigment patterns in avian plumage (27). We learned that complex patterns are not encoded directly in DNA but result from interaction between a DNA-encoded cellular property and physical-chemical principles (27). We propose a generic model to represent the progression of melanocyte lineage in regenerating feathers (fig. S15B). The evolution of the multidimensional niche topology increases patterning possibilities, allowing various regulations to be choreographed with greater temporospatial freedom in the new morphogenetic space (Fig. 4B): (i) Pigmentation can be patterned along the proximal-distal feather axis, which is also a timing axis (28). (ii) The cylindrical follicle configuration (24) creates a novel medial-lateral dimension once the feather vane opens, providing more patterning possibilities. (iii) Interactions with peripheral pulp provide a third dimension of regulation. (iv) Systemic factors, such as hormonal status or seasonal changes, add a fourth dimension of regulation (1). With variation in each dimension, the combinatorial potential of pattern complexity is enormous. In addition, variation in pigment chemistry and structural colors contributes to richer color patterns (2, 3). Although these were not studied here, we expect the patterning principles to be similar. Thus, the complexity of avian pigment patterns is enriched by co-opting mechanisms regulating simple melanocyte behavior (fig. S15).

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Supplementary Materials

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Materials and Methods

Figs. S1 to S15

References (29–38)

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Protein Equilibration Through Somatic Ring Canals in *Drosophila*

Peter F. McLean¹ and Lynn Cooley^{1,2,3*}

Although intercellular bridges resulting from incomplete cytokinesis were discovered in somatic *Drosophila* tissues decades ago, the impact of these structures on intercellular communication and tissue biology is largely unknown. In this work, we demonstrate that the ~250-nanometer-diameter somatic ring canals permit diffusion of cytoplasmic contents between connected cells and across mitotic clone boundaries and enable the equilibration of protein between transcriptionally mosaic follicle cells in the *Drosophila* ovary. We obtained similar, although more restricted, results in the larval imaginal discs. Our work illustrates the lack of cytoplasmic autonomy in these tissues and suggests a role for somatic ring canals in promoting homogeneous protein expression within the tissue.

Ring canals (RCs) are cytoplasmic bridges that form from cells with arrested mitotic cleavage furrows. They provide direct cytoplasmic connections between sibling cells and, in the *Drosophila* germ line, are necessary for the transfer of nurse-cell cytoplasm into the oocyte. In RC-containing tissues where mass transfer of cytoplasm does not occur, such as in the mammalian germ line (1–5) or several somatic tissues in *Drosophila* (6–9), the role of RCs remains poorly understood because genetic perturbation of RC proteins disrupts cytokinesis. We pursued a series of microscopy-based techniques in ovarian follicle cells (FCs) and larval imaginal discs in *Drosophila* to investigate the role of somatic RCs in protein movement between cells and across an epithelium.

In *Drosophila* egg chambers, germline cysts are encapsulated by two stem cell–derived lineages of FCs that originate in the germarium and cease mitotic divisions at stage 6; RCs, however, persist until the end of oogenesis, indicating a life span of 1 to 4 days (10–12) (Fig. 1A). Ovarian FCs exchange green fluorescent protein (GFP)–tagged endogenous proteins and the photoactivatable variant of GFP (PAGFP), which can be used to assay intercellular exchange (11) (movie S1). To evaluate the contribution of RCs to this movement, we coexpressed PAGFP (13) with the RC marker GFP::Pav (14) and photoactivated single cells. Activated PAGFP moved into neighboring follicle cells only if an intervening RC

was present, indicating protein exchange occurs only through direct cytoplasmic contact (Fig. 1, B to D). After single-cell photoactivation, we determined the size of FC syncytia by iteratively activating cells that exchanged PAGFP with the initial cell. Our analysis revealed substantial variability in syncytium size; most syncytia were between 3 and 10 cells (average of 8 cells) (Fig. 1E and movie S2), with a total observed range of 1 to 38 cells. We did not find a significant correlation between syncytium size or orientation with respect to polar axes or other morphological features of egg chambers.

Electron microscopy (EM) of somatic RCs reveals no occluding or transverse cytoskeletal structures that might indicate a mechanism for protein gating or regulated transport (11, 15, 16); therefore, we tested whether protein exchange through RCs occurs by diffusion. We quantified the change in fluorescence of FC cytoplasm after a single photoactivation event (Fig. 1F) and compared these data to a computational model of diffusion developed with the Virtual Cell (VCell, www.vcell.org) (17). Our model estimated radii to be between 0.04 and 0.11 μm (Fig. 1G and fig. S1). This is slightly smaller than radii measured by EM (0.08 to 0.14 μm) (15), which does not account for the visible geometric asymmetry or partially occluding membranous structures in somatic RCs that reduce the effective radius. We conclude that the model of diffusion correctly reports the effective radius of the intervening RC and accurately replicates the movement of PAGFP between cells.

Having observed that GFP is free to diffuse between cells, we sought to investigate the relationship of RC-based syncytia to the patchlike mosaic expression of Gal4/upstream activating sequence (UAS) transgenes in FCs (18) (Fig. 2A).

We tested the idea that mosaic patches represent isolated syncytia by conducting fluorescence loss in photobleaching (FLIP) experiments with GFP.

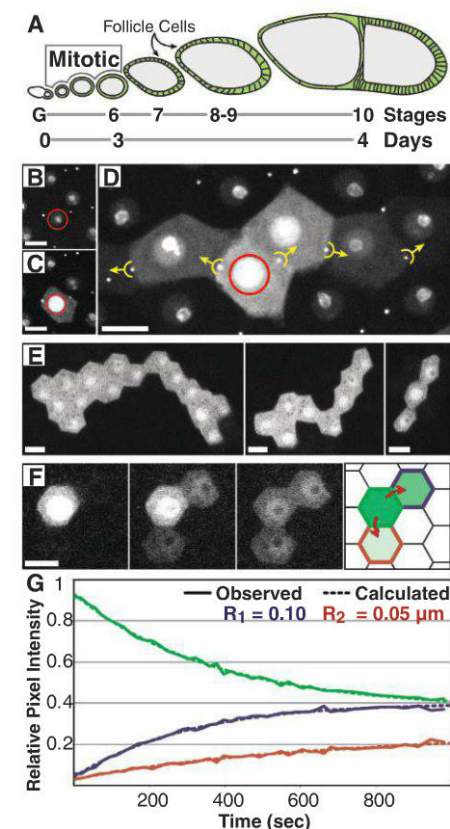


Fig. 1. Diffusion of PAGFP and syncytial organization of FCs. (A) Schematic of a *Drosophila* ovariole from the germarium (G) through a stage 10 egg chamber. The germarium cysts (gray) are encapsulated by FCs (green) that continue mitosis until stage 6, completing ~nine cell cycles. (B to D) FCs expressing GFP::Pav and Tub>mC3PAGFP were individually photoactivated (red circles), and images were collected preactivation (B), postactivation (C), and 18 min after activation (D). PAGFP only moved into cells connected by RCs (yellow markers, arrows indicate direction of movement). (E) In tissue expressing Tub>PAGFP, iterative activation revealed the extent of individual syncytia. (F and G) After a single-cell photoactivation event (F), the fluorescence intensity in FCs was measured and plotted against a model of diffusion (G). Modeled estimates of RC radii shown inset (R_1 , R_2). The cell border colors of the FC schematic (F) correspond to the data series and radii values (green indicates a donor cell; red and blue, recipient cells). Stage 10 egg chambers were used in all panels. Scale bars, 10 μm .

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Bleaching of single cells within high-expressing patches of cells caused loss of fluorescence only in neighboring cells with the same level of expression (Fig. 2B). In total, we probed 68 mosaic patch boundaries and never observed loss of fluorescence in cells of a different mosaic patch. In a complementary approach, we evaluated the extent of PAGFP diffusion within and between mosaic patches (Fig. 2C). PAGFP diffused into cells within a single patch but not into cells of a different patch. These data support our hypothesis that mosaic patches represent isolated syncytia and reflect the underlying intercellular connectivity created by RCs.

Given the nonautonomy of GFP, we conducted a fluorescent *in situ* hybridization (FISH) and immunofluorescence (IF) costain to compare the

abundance of GFP transcript and GFP protein in FCs expressing UAS-GFP. In stage 9 egg chambers, when the c855a-Gal4 driver begins expression, we found few cells with detectable levels of GFP transcript compared with the distribution of GFP protein (Fig. 2D). In stage 10 egg chambers, where c855a-Gal4 expression is strongest, we found small, dispersed clusters of two to four cells with high levels of transcript among large groups of cells with high concentrations of GFP protein (Fig. 2E and fig. S2). Thus, under the control of the c885-Gal4 driver, GFP protein is translated in a few, sparse cells and equilibrates within FC syncytia to provide protein to the majority of cells within the epithelium.

GFP is also a common marker used with flapase recognition target (FRT)-based mitotic recombination, a strategy used to generate clonal groups of genetically mutant cells within a heterozygous tissue (19). Diffusion of protein into or out of these groups of cells (clones) via RCs could compromise subsequent phenotypic analysis, so we generated a two-part labeling system

to investigate this possibility. We combined the LacO/I DNA marker, which reliably marks the recombination status of each cell (20–22), with a diffusible, recombination-induced Ubi-GFP transgene. After recombination and mitosis, GFP is reconstituted in cells containing two copies of the LacO element (2xLacO) (Fig. 3A and supplementary text). We then look for the presence of GFP in the other, LacO-negative side of the clone as evidence for diffusion through the bridging RC that connects the 2xLacO and LacO-negative cells (Fig. 3B). We refer to this method as syncytial tracing by recombination-induced transcription, or STRIT (17).

Our results revealed extensive diffusion of GFP across the two sides of FRT clones and into nonrecombinant cells. We categorized 145 isolated clones by the extent of GFP diffusion into the LacO-negative cells: no diffusion across the bridge RC (None), diffusion into part (Part) or all (Full) of the LacO-negative clone, and diffusion beyond the LacO-negative clone into nonrecombined cells (Full+) (Fig. 3, B and C). In

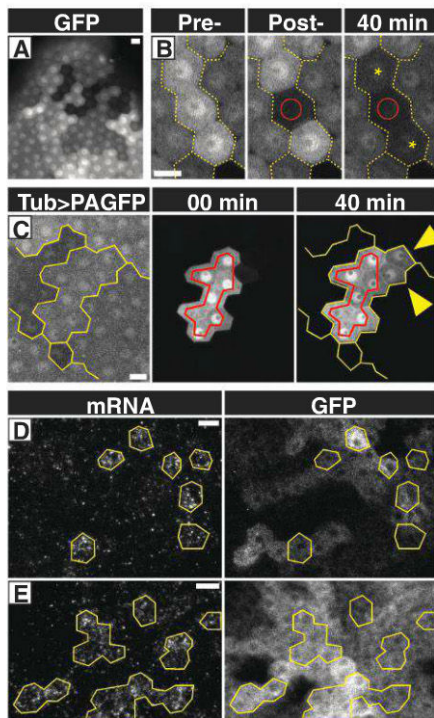


Fig. 2. Protein equilibration in FCs. (A) Stage 10 FCs expressing c855a>GFP have a mosaic, patchlike distribution of GFP. (B) FLIP analysis evaluates the intercellular connectivity of cells within patches (yellow lines indicate patch boundaries). Upon bleaching single cells (red circles), multiple cells within the patch exhibit FLIP (yellow asterisks). (C) Detection of the low, preactivation fluorescence of Tub>mC3PAGFP in stage 10 FCs reveals its mosaic, patchlike distribution. Activation (red outline) within a mosaic patch of cells (yellow outline) showed diffusion only within the patch (yellow arrowheads) and no diffusion across a boundary. (D and E) FISH/IF analysis of c855a>GFP egg chambers showed GFP transcript to be significantly more mosaic than GFP protein. Only single, isolated cells in stage 9 egg chambers (D) and small clusters of cells in stage 10 egg chambers (E) (outlined in yellow) had detectable levels of GFP transcript compared with wider distribution of GFP protein at both stages. Scale bars, 10 μ m.

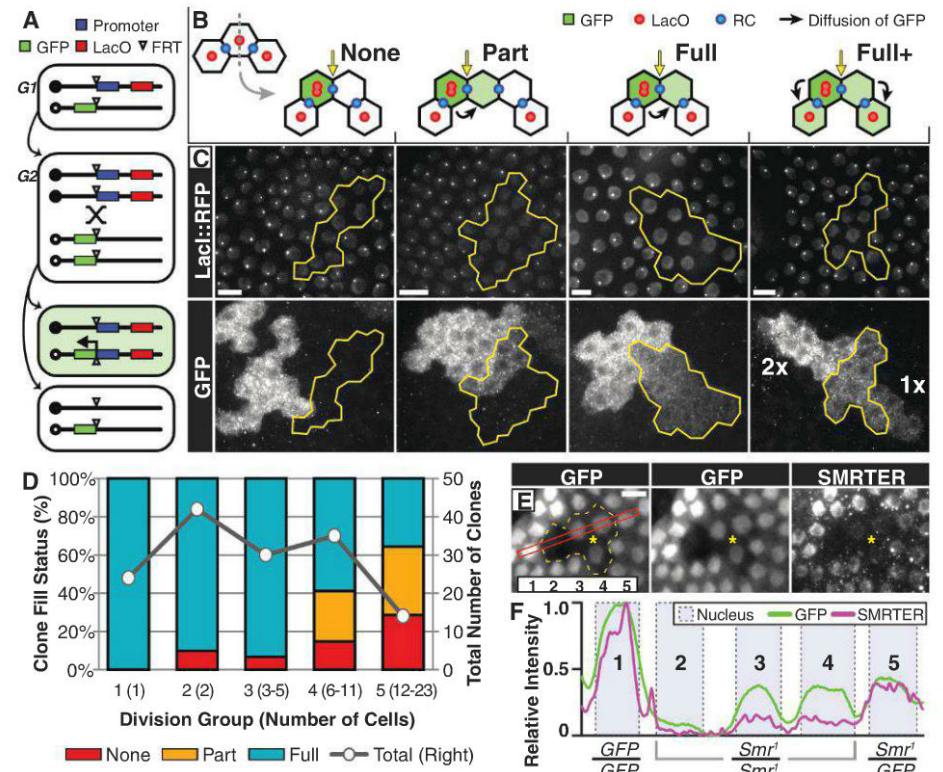


Fig. 3. Nonautonomy of mitotic clones. (A) Representation of the STRIT marking system in G1, G2, and after mitosis. One recombinant daughter contains 2xLacO and a functional GFP gene, whereas the other inherits neither. (B) Possible outcomes of STRIT clones: no GFP diffusion (None), diffusion across the bridge RC (yellow arrow) into some (Part) or all LacO-negative cells (Full), and diffusion into nonrecombined cells (Full+) through preexisting RCs. Gray line indicates plane of division. (C) Representative STRIT clones displaying varying degrees of interclonal diffusion. In Full+, GFP from 2xLacO cells (2x) is found in nonrecombined, 1xLacO cells (1x) on the far side of the LacO-negative clone. (D) Evaluation of LacO-negative STRIT clones grouped according to the degree of GFP diffusion and clone age or division group (number of cells). The total number of clones in each division group (gray series) is given on the right axis. (E) A four-cell *Smr¹/Smr²* clone (outlined in yellow) in which three cells (around yellow asterisk) contain detectable levels of GFP and Smrter proteins. The red rectangle indicates the region quantified in (F), and cells in the region are numbered (inset). (F) Cells 3 and 4 contain lower but detectable levels of both GFP and Smrter, indicating diffusion into the *Smr¹/Smr²* clone. Scale bars, 10 μ m.

total, 90% of LacO-negative clones contained GFP, suggesting that nearly all observable RCs in FCs permit intercellular diffusion.

We further categorized our results by the number of cell cycles since clone formation (division group) as a measure of the clone's age (Fig. 3D). We observed an increasing fraction of GFP-negative (None) and Part clones in older STRIT lineages, indicating a loss of functional bridge RCs (supplementary text). These data support an estimated RC half-life of 10 cell cycles, or ~4 days, which is close to the ~4.5-day life span of FCs, suggesting that the majority of RCs remain functional throughout oogenesis. We also counted the appearance of nonfunctional connections within LacO-negative clones of the Part-filled group to be about 15% of the total number of divisions (27/179). Because this value is based on the diffusion of GFP and therefore includes possible nonfunctional RCs still visible by microscopy, the complementary 85% represents an estimate of the number of functional RCs. In sum, these data support previous reports that ~90% of FC divisions form RCs (11) and also suggest that an additional ~5% of RCs visible by confocal microscopy no longer connect cells. The functional 85% of RCs directly connect the cytoplasm of cells within their lineage and form many syncytial groups in which diffusible protein is free to equilibrate. Interclonal diffusion of markers (i.e., GFP) or proteins (11) can result in ambiguous clonal boundaries and complicate the interpretation of phenotypes, as evidenced by diffusion of both GFP and Smrter protein into *Smr^l* cells (Fig. 3, E and F, and fig. S4).

EM and confocal microscopy have also revealed somatic RCs in *Drosophila* larval imaginal discs (7, 8, 11, 16). We determined that 60 to 80% of cellular divisions are represented by GFP::Pav-labeled structures in third instar wing

discs, equaling about 1500 to 3000 potential RCs within the wing blade (fig. S5). We assayed intercellular connectivity in the wing disc by photoactivation of PAGFP and STRIT clonal analysis and observed evidence of intercellular diffusion with both techniques (Fig. 4, A to C, and fig. S6). Although technical challenges of imaginal disc tissue made reliable identification of intercellular diffusion more difficult, we estimate that <20% of cells showed intercellular diffusion with PAGFP and <10% of STRIT clones showed diffusion across the bridge RC. Additionally, we observed GFP in LacO-negative clones only up to two cells in size, suggesting that bridge RCs have a short functional life span (less than two cell cycles). The perdurance of nonfunctional RCs in the tissue could explain the abundance of GFP::Pav structures observed by microscopy. In support of this hypothesis, EM of the wing imaginal discs revealed both intact RCs and many examples of engulfed, burst, and degrading RCs (Fig. 4, D and E, and fig. S7). Together, these results suggest that RCs allow intercellular diffusion in imaginal discs as they do in FCs, but because the number of functional RCs in imaginal discs is low, they are unlikely to substantially alter large-scale pattern formation.

Our results provide strong evidence that somatic RCs act as direct intercellular channels through which cytoplasmic proteins can diffuse. In the ovarian FCs, RCs remain functional for several days and result in the formation of syncytial groups that allow equilibration of proteins between cells and, in many cases, across mitotic clones. The incomplete autonomy of mitotic clones in FCs urges caution when interpreting mitotic clone data, because clonal markers or proteins of interest may not accurately report the genotypes of cells. We also show that transcriptional disparities underlie the observed mosaicism of Gal4/

UAS-GFP within the follicular epithelium and that they are much greater than is evidenced by protein concentration, which equilibrates among the cells of each syncytial group. For proteins and protein complexes that are unable to diffuse between cells, including several ribosomal proteins, endogenous protein concentrations are visibly mosaic (11). Thus, the ability to equilibrate levels of some proteins in syncytial clusters may benefit FCs by allowing them to compensate for transcriptional variations across the epithelium.

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Supplementary Materials

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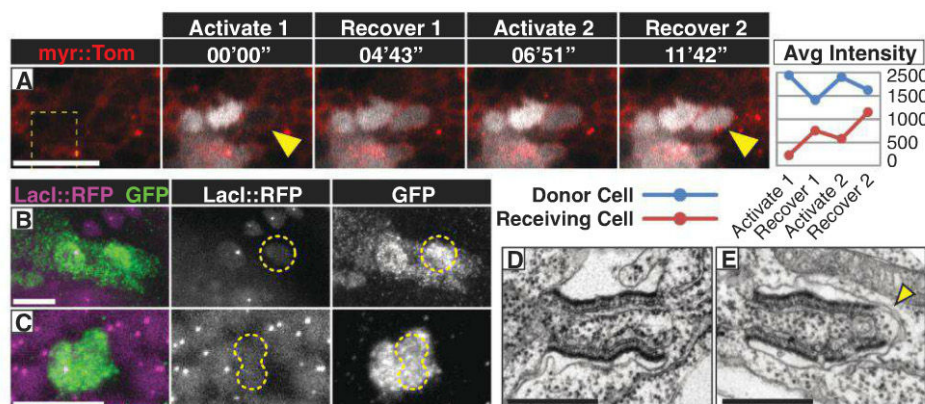


Fig. 4. Intercellular diffusion in the wing imaginal disc. (A) Photoactivation of *UAS-mC3PAGFP* (white channel) in a region of the wing blade (dashed line) reveals rapid intercellular diffusion (yellow arrowheads). Two consecutive activation and recovery events are shown and quantified on the right (average pixel intensity). Intercellular exchange is evidenced by the decrease in cytoplasmic fluorescence in the activated donor cell during the recoveries while the receiving cell shows an increase. (B and C) STRIT clones in wing imaginal discs show diffusion of GFP into small LacO-negative clones (yellow outlines). (B) One-cell clone in the peripodial epithelium; (C) two-cell clone in the wing-blade epithelium. Scale bars, 10 μ m. (D and E) EMs of RCs in the wing imaginal disc. Many longitudinally sectioned RCs connect cells (D), but about half do not. (E) Engulfed RC with a membrane cap at one end (yellow arrowhead). Scale bars, 500 nm.

Quantum Coherent Energy Transfer over Varying Pathways in Single Light-Harvesting Complexes

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The initial steps of photosynthesis comprise the absorption of sunlight by pigment-protein antenna complexes followed by rapid and highly efficient funneling of excitation energy to a reaction center. In these transport processes, signatures of unexpectedly long-lived coherences have emerged in two-dimensional ensemble spectra of various light-harvesting complexes. Here, we demonstrate ultrafast quantum coherent energy transfer within individual antenna complexes of a purple bacterium under physiological conditions. We find that quantum coherences between electronically coupled energy eigenstates persist at least 400 femtoseconds and that distinct energy-transfer pathways that change with time can be identified in each complex. Our data suggest that long-lived quantum coherence renders energy transfer in photosynthetic systems robust in the presence of disorder, which is a prerequisite for efficient light harvesting.

Highly efficient excitation energy transfer is a key step in the initial light-driven processes of photosynthesis and takes place in sophisticated supramolecular assemblies, so-called pigment-protein complexes (1, 2). Much work has been devoted to revealing the underlying mechanisms and to understanding the spatial and energetic organization of the pigment molecules involved (1–8). A particularly intriguing question that recently emerged concerns the impact of quantum coherence on promoting the efficiency and the directionality of ultrafast energy transport in photosynthesis. Although the presence of this quantum effect is now generally accepted (9), many open questions associated with this

phenomenon remain, including how light-harvesting antennae evolved to be robust against perturbations and thermal disorder under physiological conditions and whether quantum coherent transport can help to optimize the energy flow despite the presence of disorder. Owing to the large structural and electronic heterogeneity of photosynthetic antenna proteins (10–12), such issues are not testable by conventional femtosecond spectroscopy (2–5).

We addressed these questions by using an ultrafast single-molecule technique (13, 14) to study a prototypical antenna protein, the light-harvesting 2 (LH2) complex from the purple bacterium *Rhodospseudomonas acidophila*. In this assembly, 27 bacteriochlorophyll a (Bchl a)

molecules are arranged in two concentric rings (Fig. 1A) that give rise to the characteristic near-infrared absorption bands (Fig. 1B), labeled according to their absorption maxima, B800 (with 9 weakly coupled Bchl a pigments) and B850 (with 18 strongly coupled Bchl a). Because the B800–B850 transfer within these complexes is governed by an intermediate electronic coupling (7, 8), we are particularly interested in the role of quantum coherence in the transport between these energy eigenstates.

The femtosecond coherent B800–B850 dynamics in single LH2 complexes were studied by a specific two-color experiment. A Fourier-limited 15-fs pulse was phase-shaped into two time-delayed transform limited pulses with different carrier frequencies that overlap with the B800 and B850 absorptions of LH2, respectively (Fig. 1C). This was achieved by selectively applying a linear phase ramp to the appropriate spectral band in a pulse shaper (Fig. 1B, green line), which gives full control over the delay time, Δt , as well as the relative carrier envelope phase, $\Delta\phi$, between both pulses. We detected the fluorescence signal, that is, the total population probability of the lowest-

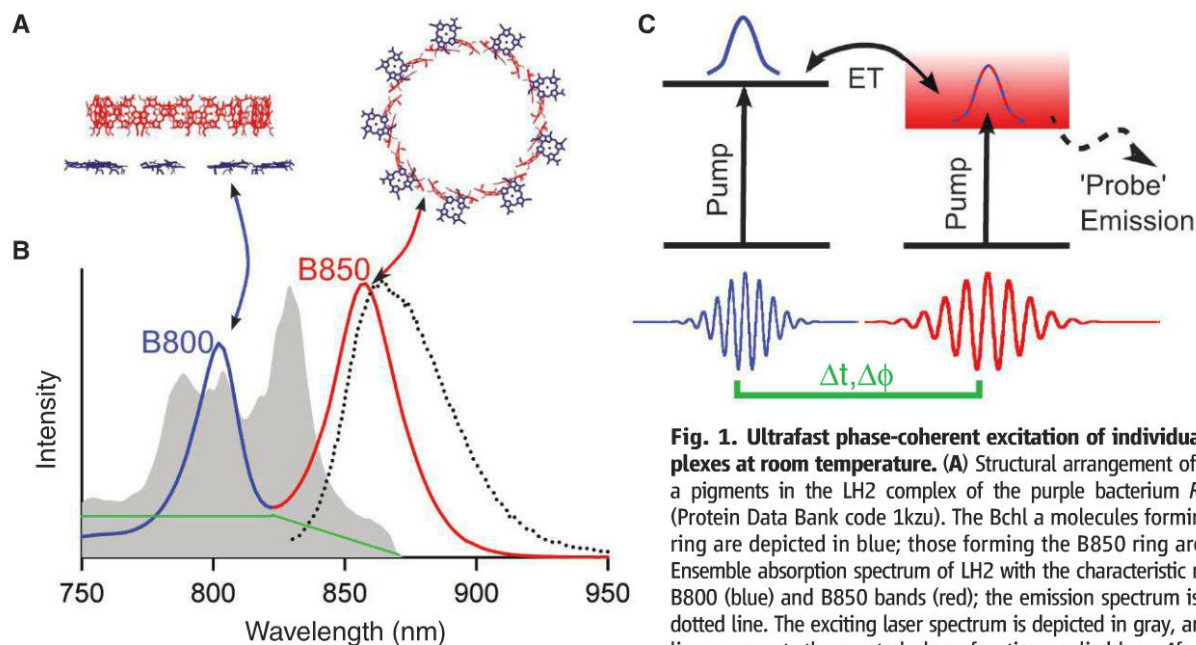


Fig. 1. Ultrafast phase-coherent excitation of individual LH2 complexes at room temperature. (A) Structural arrangement of the 27 Bchl a pigments in the LH2 complex of the purple bacterium *R. acidophila* (Protein Data Bank code 1kzu). The Bchl a molecules forming the B800 ring are depicted in blue; those forming the B850 ring are in red. (B) Ensemble absorption spectrum of LH2 with the characteristic near-infrared B800 (blue) and B850 bands (red); the emission spectrum is shown as a dotted line. The exciting laser spectrum is depicted in gray, and the green line represents the spectral phase function applied by a 4f-pulse shaper. (C) Concept of the experiment: Single LH2 are excited by a two-color pulse pair with Δt and $\Delta\phi$ generated by applying the spectral phase function. The first pulse (blue) creates an excitation in the B800 band. After energy transfer (ET) to the B850 band, the second time-delayed pulse (red), resonant with the B850, modulates the population transfer to the B850 excited states by quantum interference and thus changes the probe signal, the spontaneous emission from a single complex.

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energy and emitting B850 exciton state after interaction with both pulses. The spectral amplitudes were the same for all (Δt , $\Delta\phi$) combinations in our experiments (for details, see materials and methods in the supplementary materials).

In a first experiment, we varied the delay time Δt while keeping the relative carrier phase $\Delta\phi$ constant. Individual LH2 complexes feature pronounced oscillations in their emission as a function of Δt up to at least 400 fs, as shown for two representative examples (Fig. 2, B and C). Although both complexes exhibit oscillation periods of around 200 fs and contrasts of about 15% (ratio between maximum and minimum signal), there are differences in their ultrafast B800-B850 transfer dynamics. In order to quantify this femtosecond response, we retrieved the oscillation periods, T , from the delay traces by a cosine fit to the data. The resulting histogram (Fig. 2D) features a wide distribution between 140 and 400 fs with a maximum near 200 fs. This broad spread in T demonstrates the large structural and electronic disorder between complexes that is caused by the heterogeneous local dielectric protein environment (15, 16). This disorder is typically hidden in ensemble experiments but has a substantial impact on the specific B800-B850 transport within each complex. Because the dynamics of this transfer depend on the site energies as well as on the mutual orientations and distances of the involved pigments, that is, on their particular electronic couplings, these different ultrafast responses imply that each complex features a distinct transfer pathway characterized by its period T (see fig. S3 and accompanying text for more details on resolving this heterogeneity and different pathways). On the basis of an analytical treatment, the distribution of T is consistent with an average electronic coupling of $J \approx 50 \text{ cm}^{-1}$ between B800

and high-energy B850 states (see supplementary materials), which is in agreement with ensemble experiments and theory (3, 5) and indicates that optically dark high-energy B850 exciton states are involved in these first transfer steps.

We attribute the oscillations in Fig. 2, B and C, to quantum interference between two excitation pathways that populate the same target state, the emitting lowest-energy B850 level. Single complexes are first excited with a broadband short-wavelength pulse ($\Delta t > 0$) that is resonant with B800 and thus creates an excitation in this band. This excitation evolves under field-free conditions during the time interval Δt . In particular, electronic coupling mixes B800 and B850 states, and the total wave function can be expressed as $|\Psi\rangle = c_{\text{B800}}|\Psi_{\text{B800}}\rangle + c_{\text{B850}}|\Psi_{\text{B850}}\rangle$; that is, an electronic coherence between B800 and B850 is induced by the electronic B800-B850 coupling. After relaxation to optically allowed low-energy B850 excited states, the delayed longer wavelength pulse then either enhances or reduces the population of the emitting B850 level by quantum interference. This effect depends critically on the survival time of quantum coherence in the system (see fig. S2 and accompanying text), for which several mechanisms have been proposed (16–19). In contrast, if the electronic coherence decayed rapidly within ~ 50 fs, as usually expected for a disordered system at room temperature, we would observe a constant emission as a function of Δt , except for a short interval $\Delta t \leq 50$ fs where phase memory is present (14).

The electronic nature of the coherences giving rise to the oscillatory delay traces (Fig. 2, B and C) was confirmed by control experiments on LH2 (fig. S6) and by recent two-dimensional spectroscopy on isolated (20) and protein-bound Bchl *a* pigments (21–24). Consequently, the persistent

oscillations in the traces in Fig. 2, B and C, are a signature that electronic quantum coherences between B800 and B850 survive for an unexpectedly long time, at least 400 fs, in individual LH2 complexes under physiological conditions. Similar quantum effects have been reported by Lee *et al.* and Harel *et al.*, who observed coherences between electronic eigenstates in light-harvesting antenna proteins by ensemble photon-echo methods (21, 25).

A crucial question concerns the role of coherence in the robustness of energy transport against external perturbations, such as conformational fluctuations, that occur in LH2 on time scales of seconds (10) and modify the electronic couplings between pigments on such slow time scales. To investigate this issue, we resolved phase changes in the energy transfer by recording the fluorescence of individual complexes as a function of the relative carrier envelope phase $\Delta\phi$ at constant Δt . The delay time was chosen to be longer than 100 fs to avoid pulse-overlap effects but well below 400 fs to remain in the coherent regime. This phase-cycling measurement (Fig. 3, red symbols) features a full sinusoidal period of the emission upon a relative phase change of more than 2π at $\Delta t = 150$ fs. A comparison with the reference signal from the same complex (Fig. 3, open black circles), taken repeatedly with a Fourier-limited pulse encompassing the full laser spectrum, shows that the excitation probability is enhanced by 15% for a relative phase of about 0.5π (and reduced by 15% for a relative phase of $\sim 1.5\pi$). This observation demonstrates weak-field phase control of the quantum interference between the excitation pathways to B800 and B850 excited states or, in other words, phase control of the efficiency of the coherent contribution to the functionally important B800-B850 transfer pathway.

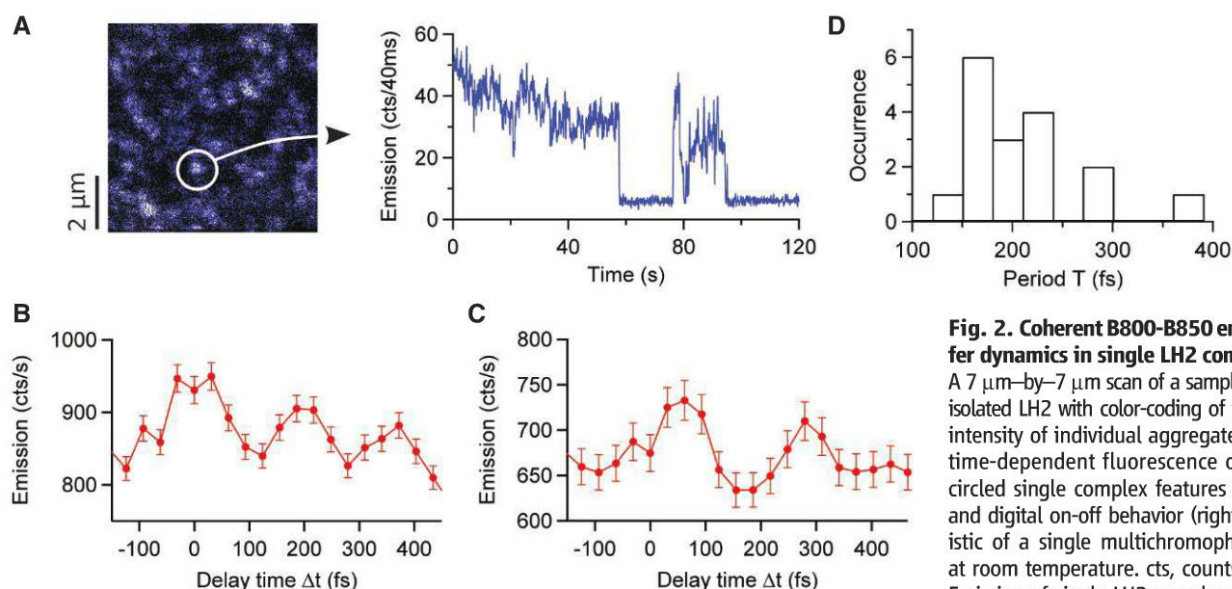


Fig. 2. Coherent B800-B850 energy transfer dynamics in single LH2 complexes. (A) A 7 μm -by-7 μm scan of a sample containing isolated LH2 with color-coding of the emission intensity of individual aggregates (left). The time-dependent fluorescence of the white circled single complex features fluctuations and digital on-off behavior (right) characteristic of a single multichromophoric system at room temperature. cts, counts. (B and C) Emission of single LH2 complexes as a function

of the interpulse delay time with constant relative carrier envelope phase, demonstrating coherent population oscillations between B800 and B850 bands with very long coherence times of hundreds of femtoseconds. Error bars indicate ± 1 SD based on a total number of $N = 4 \times 10^3$ (B) and 1.5×10^3 (C) photons. (D) Histogram of the oscillation period T retrieved from traces as shown in (B) and (C).

Such efficient phase control indicates that the time scale for B800-B850 population transfer (1, 2) is comparable to that for dissipative interactions with the local (protein) bath (26, 27).

Notable variations in the ultrafast response of single complexes were tracked with our rapid phase-cycling approach (Fig. 4 and fig. S5). As an example, Fig. 4A presents the raw data from an individual aggregate. Each block with about 8-s measurement time represents a phase scan from 0 to 2π at a constant interpulse delay of 100 fs. Whereas the (temporal) average over the entire 120-s measurement (Fig. 4B, green symbols) is basically identical to the reference signal (open black symbols) and does not reveal phase-sensitive features, analyzing shorter time intervals of the data gives a completely different picture (Fig. 4C): The red circles depict the emission as a function of phase for the first ~35 s of observation time, and the blue circles show the fluorescence at later times. The observed jump of the oscillation phase by about π demonstrates a change in the ultrafast response of this single complex after some 10 s. This phase jump must result from a change of the accumulated phase of the relaxing excitation (supplementary materials), because the experimental conditions were the same throughout the entire measurement. This jump implies a modification of the energy transfer pathway from B800 toward possibly different low-energy, optically accessible B850 excited states (e.g., the ± 1 exciton levels) caused by subtle structural rearrangements in the complex (10, 11) that alter mutual distances and angles between the Bchl *a* pigments as well as their site energies because of different local interactions. The amplitude of the phase-dependent modulation remains basically constant, although the phase relation is inverted, which indicates persistent coherence even for changing transfer pathways.

Both ensemble and temporal averaging washes out all features observed in the subsets of the

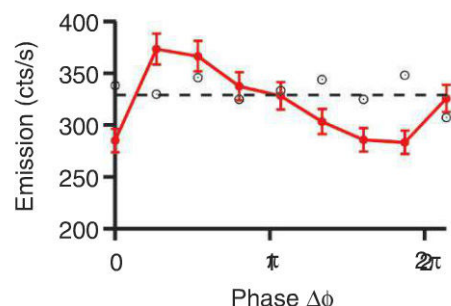


Fig. 3. Coherent phase control of the population transfer to the excited states of a single LH2 ring. Fluorescence signal of a single LH2 complex as a function of $\Delta\phi$ at $\Delta t = 150$ fs (red). The emission features a 15% enhancement (reduction) compared with the reference signal (black symbols), that is, the emission upon excitation with a single transform-limited pulse covering the entire 120 nm spectral band width. Error bars, ± 1 SD based on a total number of $N = 10^3$ photons.

data, as illustrated in the total average in Fig. 4B and fig. S5 (open green symbols). Hence, experiments integrating over too long time scales or on ensembles of many complexes would lead to the unjustified conclusion that phase memory was already completely lost within $\Delta t = 100$ fs. These data demonstrate that only single-molecule detection allows all subtle details of a complex system in the presence of thermal disorder to be revealed.

Persistent quantum coherences in photosynthetic complexes were previously attributed to correlated nuclear motions of the pigments' local dielectric environments that give rise to long-lived site energy cross-correlations (21, 22, 25, 28), whereas subsequent molecular dynamics simulations have failed to reproduce such correlated protein fluctuations (16, 18). However, this latter work indicates that coherences in antenna complexes can still survive hundreds of fs as long as the electronic coupling between pigments is strong enough and does not change on the relevant time scales (16). Moreover, it has very recently been suggested that interactions with discrete bath modes featuring energies similar to energy differences of exciton states may help to sustain long-lived coherent energy transport (17). For the biological function of LH2, it is the interplay between this persistent coherence and energy

dissipation by interactions with the surrounding bath (19, 29–31) that rapidly directs the excitation energy toward the lowest-energy B850 target levels, from which further transfer to adjacent LH2 or LH1 occurs. Dissipative interactions, on one hand, stabilize the initially created electronic excitations in lower-energy states on sub-ps time scales to create the ultrafast energy funnel to bottom B850 states and to prevent relaxation along loss channels. On the other hand, quantum coherences survive long enough to allow averaging over local inhomogeneities of the rough excited-state energy landscape and thus to avoid trapping in local minima. The highest efficiencies for this environmentally assisted quantum transfer in photosynthetic light-harvesting complexes have been found for comparable time scales of population transfer and dissipative relaxation (29–31). This requirement is fulfilled for the B800-B850 transfer, as shown in Figs. 3 and 4, by efficient weak-field phase control of the one-photon transitions into the B800 and B850 excited states.

An intriguing feature of our observations is that the quantum beats are rather well-defined compared with ensemble data and can be described by only a single oscillatory component. Hence, it seems that in each specific LH2 complex only one energy transfer pathway at a time is present. Because a rather large number of pig-

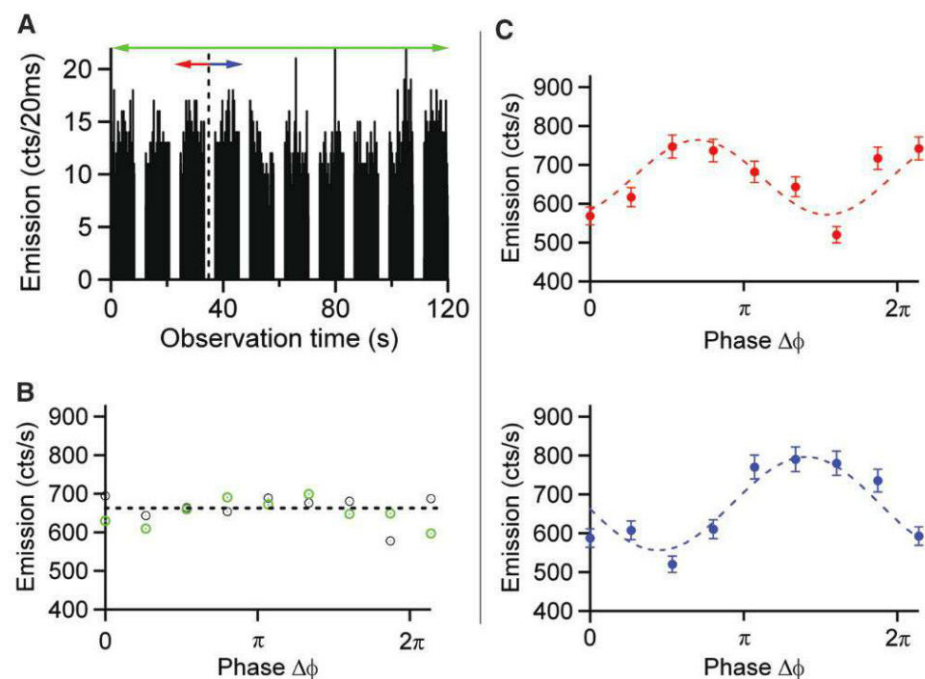


Fig. 4. Time-varying coherent energy transfer pathways. (A) Raw data from a single LH2 complex as a function of observation time: Each block represents a phase scan from 0 to 2π at $\Delta t = 100$ fs, interleaved with reference measurements using a single Fourier-limited pulse. (B) Reference signal (black symbols) and LH2 emission as a function of $\Delta\phi$ temporally averaged over all 10 raw data blocks (green symbols). The dashed line is a guide to the eye representing the average signal. (C) Phase dependence of the LH2 emission averaged over the blocks left (red symbols, top) and right (blue symbols, bottom) of the dashed line in (A), considering only blocks with a constant reference signal. The red and blue dashed lines are cosine fits to the corresponding data, revealing a phase jump of about π after about 35 s of observation time. This is attributed to a modified energy transfer pathway within this single complex. Error bars, ± 1 SD based on a total number of $N = 2 \times 10^3$ (top) and 1.5×10^3 (bottom) photons.

ments and thus possible pathways exist per ring, this is likely to be the optimal pathway for the particular geometrical and electronic structure, that is, for the specific electronic couplings. Moreover, the phase jumps observed for some assemblies (Fig. 4 and fig. S5) indicate that long-lived coherences also may provide flexibility to adapt to modifications of the (local) electronic structure and to find efficient new energy-transfer pathways within a complex. In other words, long-lived coherences contribute to the necessary robustness against external perturbations and disorder that are ubiquitous in biological systems at physiological temperatures. In this respect, the biological function of these complexes, light absorption and energy funneling toward the reaction center, is optimized for each individual aggregate, and long-lived quantum coherences herein play an important role.

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Supplementary Materials

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Structure of Parkin Reveals Mechanisms for Ubiquitin Ligase Activation

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Mutations in the *PARK2* (*parkin*) gene are responsible for an autosomal recessive form of Parkinson's disease. The parkin protein is a RING-in-between-RING E3 ubiquitin ligase that exhibits low basal activity. We describe the crystal structure of full-length rat parkin. The structure shows parkin in an autoinhibited state and provides insight into how it is activated. RING0 occludes the ubiquitin acceptor site Cys⁴³¹ in RING2, whereas a repressor element of parkin binds RING1 and blocks its E2-binding site. Mutations that disrupted these inhibitory interactions activated parkin both in vitro and in cells. Parkin is neuroprotective, and these findings may provide a structural and mechanistic framework for enhancing parkin activity.

Parkinson's disease (PD) is a common neurodegenerative disease characterized by severe motor and nonmotor symptoms. More than 120 mutations in *PARK2* (*parkin*) have been shown to cause autosomal recessive PD, with

point mutations found in every domain of the protein (1–3). The parkin protein is a RING-in-between-RING (RBR) E3 ubiquitin ligase (4) that exhibits low basal activity in vitro (5). Parkin has been implicated in a range of biological processes, including autophagy of damaged mitochondria (mitophagy), cell survival pathways, and vesicle trafficking (6, 7). The activity of the PD-associated mitochondrial kinase PINK1 is required for parkin activation in mitophagy (8–12). Parkin consists of a ubiquitin-like (Ubl) domain and a 60-amino acid linker followed by RING0, a zinc finger unique to parkin (13), and three additional zinc finger domains characteristic of the RBR family

(Fig. 1A). RBR ligases such as parkin, HOIL-1L interacting protein (HOIP), and Ariadne/HHARI use a RING-HECT hybrid mechanism (14–16) whereby ubiquitin forms a thioester intermediate with a cysteine side chain in RING2 before being transferred to the primary amino group of a substrate to form an isopeptide bond. How RBR ligases accomplish these two reactions is unknown.

We determined the crystal structure of rat parkin, obtaining a low-resolution structure of the full-length protein and a 2.8 Å resolution structure of a C-terminal fragment (amino acids 141 to 465) (Fig. 1B, figs. S1 and S2, and table S1). Collectively, the structures show that parkin forms a rigid core of the RING0, RING1, and RING2 domains stabilized by several key hydrophobic interactions (Fig. 1C). This core functions as a scaffold for interactions with the remaining domains of parkin. The in-between-RING (IBR) domain is attached through a flexible linker; its position varies by up to 13 Å between different chains in the structure of the C-terminal fragment (fig. S2B). The N-terminal Ubl domain is bound to RING1 through the hydrophobic surface centered around Ile⁴⁴, consistent with the reported interaction between the Ubl and the C terminus (5). This surface of the Ubl also binds ubiquitin-interacting motifs (17–19) (UIMs) and SH3 domains (20), implying that the Ubl must dissociate from RING1 in order to bind these partners. The long linker following the Ubl is not visible in the crystal structure, but a fragment of the second linker between the IBR and RING2 domains is visible. This fragment contains an α helix bound to RING1, called REP (repressor element of parkin).

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Small-angle x-ray scattering (SAXS) analysis revealed that both full-length parkin and R0-RBR parkin adopt the same conformation in solution as in the crystals (Fig. 1D and fig. S3).

The structure explains a large number of mutations associated with PD (Fig. 1E). The mutations can be grouped into three classes: mutations such as Arg⁴² → Pro, Lys²¹¹ → Asn, Cys²¹² → Tyr, Cys²⁸⁹ → Gly, Thr³⁵¹ → Pro, and Cys⁴⁴¹ → Arg that disrupt zinc coordination or protein folding; mutations such as Gly⁴³⁰ → Asp and Cys⁴³¹ → Phe that affect the catalytic site; and mutations that affect protein contacts. For example, in the Ubl domain, the mutation Arg³³ → Gln could interact with the adjacent IBR domain. The position and flexibility of the IBR is probably important for parkin's function: The mutation Gly³²⁸ → Glu in the hinge between RING1 and IBR causes PD, possibly via loss of conformational flexibility. In the crystal structure, the Gly³²⁸ backbone torsion angles ($\phi \sim 90^\circ$; $\psi \sim 20^\circ$) are incompatible with glutamate. Some mutations such as Asp²⁸⁰ → Asn and Thr⁴¹⁵ → Asn may affect as-

pects of parkin function other than its stability, catalysis, or interdomain contacts.

The parkin zinc finger domains fold with distinct topologies. RING1 is the only domain with a classical C3HC4 cross-brace zinc coordination topology typical of other RING fingers (Fig. 1F and fig. S4). Modeling based on the structures of five E2-RING complexes reveals the E2 binding site on RING1 (fig. S5). The other domains are atypical. RING0 binds two coordinated zinc atoms at each extremity of the domain with a hairpin. The IBR domain is similar to the reported nuclear magnetic resonance (NMR) structure of the isolated domain (21) (fig. S2D). The RING2 domain has the same topology as the IBR domain and coordinates two zinc atoms in a sequential fashion (Fig. 1F). Two of the zinc coordinating residues in RING2, Cys⁴⁵⁷ and His⁴⁶¹, are not conserved in other RBR proteins (fig. S6) but are well conserved in parkin (fig. S7). Moreover, these two residues are part of a helix that mediates multiple interactions with a hydrophobic groove in RING0 (Fig. 2A).

A number of structural features suggest that the observed conformation is autoinhibited. First, the catalytic Cys⁴³¹ is occluded by the RING0-RING2 interactions (Fig. 2B). Second, modeling of the E2 binding site on RING1 suggests that it is located beside the Ubl binding site and blocked by the REP linker (Fig. 2C and fig. S5). The REP is well conserved across parkin orthologs (Fig. 2D) and forms a two-turn helix that is held in place by Trp⁴⁰³, whose indole group inserts into a pocket in RING1 in part formed by the side chain of Arg²³⁴ (Fig. 2C), a residue mutated to glutamine in PD. Third, parkin must undergo a conformational change to mediate the transfer of ubiquitin, because the active-site cysteines on the E2 and parkin are ~ 50 Å apart in the E2-parkin models (fig. S5B).

To assess the role of the Ubl domain and the RING0-RING2 and REP-RING1 interactions on parkin activity, we tested a series of deletions and single-site mutants in autoubiquitination assays. Wild-type parkin displayed weak but detectable activity, monitored by the appearance of high-

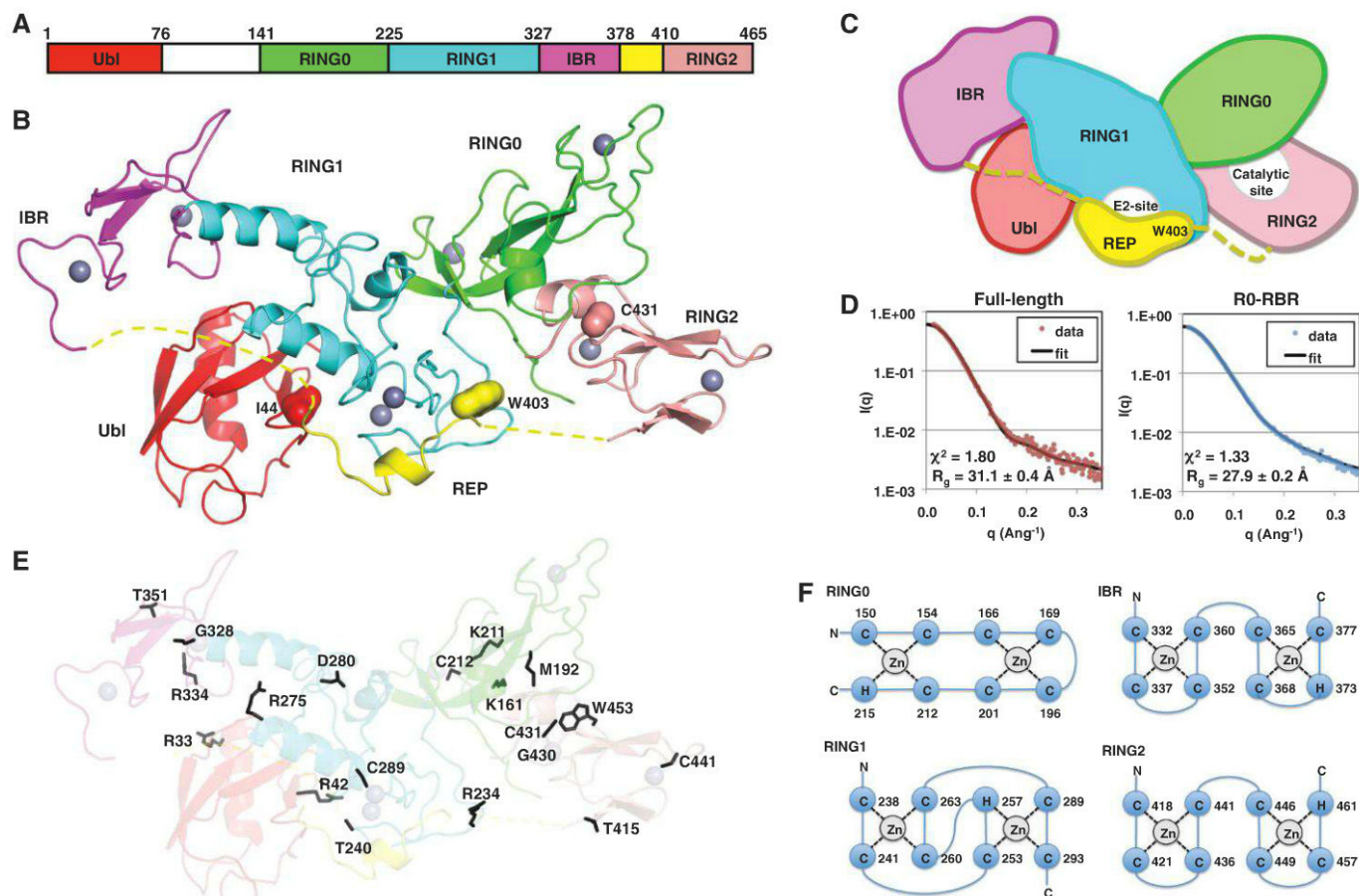


Fig. 1. Structure of parkin. (A) Primary structure and domains of parkin. (B) Cartoon representation of the full-length structure. The repressing REP α helix is surrounded by unstructured regions (yellow dashed lines). Zinc atoms are shown as gray spheres. (C) Schematic representation of the full-length structure showing the occluded E2 binding and catalytic sites. (D) SAXS data were obtained for both full-length and R0-RBR (amino acids 141 to 465) parkin in solution. The data were fitted to single chains in each crystal structure. (E) Mapping of human PD mutations onto the structure. Position 240 is alanine in

rat parkin. (F) Topology of zinc finger domains in parkin. Cysteine or histidine zinc ligands are shown in blue circles connected by lines from the N terminus to the C terminus. The RING1 domain displays the cross-brace motif characteristic of RING domains. RING0 displays a hairpin topology, whereas IBR and RING2 display sequential topologies. Single-letter abbreviations for amino acid residues: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

molecular weight polyubiquitinated proteins and the loss of unmodified parkin (Fig. 2, E and F). This activity required E1, E2, and ubiquitin (Fig. 2F). Autoubiquitination assays with the K0 ubiquitin variant, where all lysines were mutated to arginines, confirmed that the high-molecular weight species were polyubiquitin chains (fig. S8A). Mutation of different unliganded cysteines in parkin confirmed the unique role of Cys⁴³¹ in the catalysis of ubiquitination (fig. S8B) (14). The majority of PD mutations tested (Lys¹⁶¹ → Asn, Met¹⁹² → Val, Asp²⁸⁰ → Asn, Gly³²⁸ → Glu, Glu⁴⁴⁴ → Gln) reduced activity (Fig. 2E). Mutation of His⁴³³, located next to the active site, Cys⁴³¹ (Fig. 2A), strongly reduced activity (Fig. 2E); this finding suggests that His⁴³³ could assist

Cys⁴³¹ in catalyzing ubiquitin transfer from a charged E2 or in the downstream acyl transfer reaction onto a substrate lysine. Mutation of residues in the predicted E2 binding site (Ala²⁴⁰ → Arg, Thr²⁴² → Ala, Asp²⁴³ → Ala) all abolished parkin autoubiquitination activity (Fig. 2E). The Ubl has been reported to mediate parkin autoinhibition (5), and its position next to the E2 binding site and the REP linker also suggested this (Fig. 2C). Surprisingly, deletion of the Ubl, either alone or together with the following linker, had little effect on parkin activity, regardless of whether the proteins were tagged with glutathione-S-transferase (GST) at their N terminus (Fig. 2, F and G, and fig. S9). In contrast, the additional deletion of RING0 massively derepressed parkin

activity (Fig. 2, F and G, and fig. S9). Further supporting the role of RING0 in autoinhibition, point mutations in RING0 (Phe¹⁴⁶ → Ala) or RING2 (Phe⁴⁶³ → Ala) that had been predicted to disrupt the RING0-RING2 interface both increased parkin activity (Fig. 2, F and G, and fig. S9). Moreover, mutation of the zinc ligand Cys⁴⁵⁷ also activated parkin, confirming that the integrity of the C-terminal helix in RING2 is required to maintain the autoinhibition of parkin (fig. S8). To test the role of the REP-RING1 interface, we mutated Trp⁴⁰³ to alanine. The resulting W403A mutant also showed increased autoubiquitination activity, thereby confirming that the REP effectively represses the ligase activity of parkin. Thus, the REP and RING0 domains play a preeminent

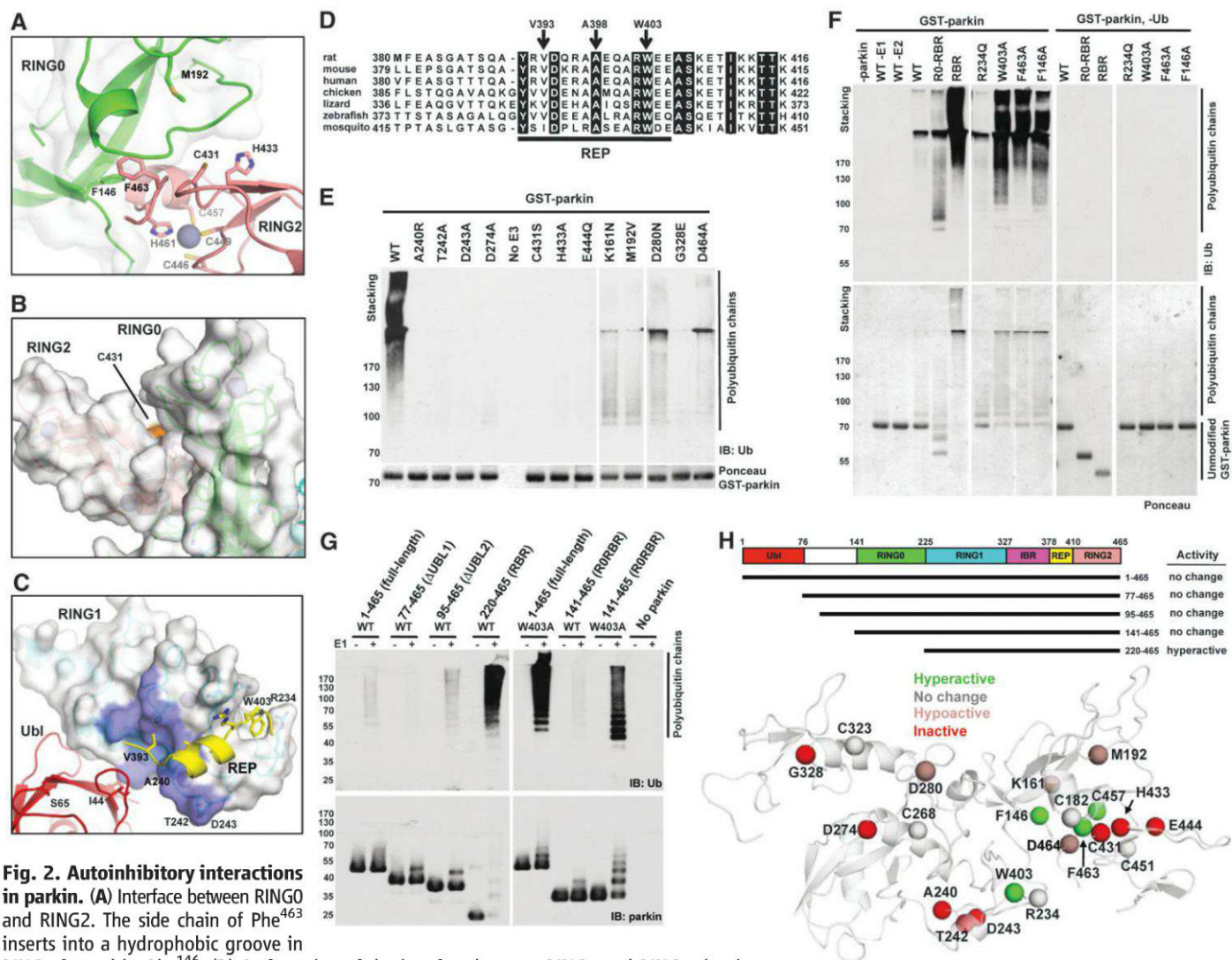


Fig. 2. Autoinhibitory interactions in parkin.

(A) Interface between RING0 and RING2. The side chain of Phe⁴⁶³ inserts into a hydrophobic groove in RING0 formed by Phe¹⁴⁶. (B) Surface view of the interface between RING0 and RING2 showing occlusion of the active site, Cys⁴³¹ (orange). (C) Interface of RING1 (cyan), Ubl (red), and the REP motif (yellow). The predicted UbcH7 binding site on RING1 is colored violet. The binding site was determined by structural overlay with the cCbl-RING-UbcH7 crystal structure (PDB ID: 1fbv) (29). (D) Sequence alignment of the REP sequence in parkin from different species. Invariant residues are shown as white letters on a black background. (E) Autoubiquitination assays with mutated GST-parkin constructs (WT, wild type). Reactions were performed with 1 μ M GST-parkin and 2 μ M UbcH7 and incubated for 3 hours at 37°C. (F) Activation of parkin E3 ligase activity by mutagenesis. Reactions were performed as described above. Autoubiquitination was followed by the appearance of high-molecular weight smear in both the stacking and resolving sections of the blots, and by the loss of unmodified parkin in the Ponceau blot. (G) Autoubiquitination assays with untagged parkin constructs. Reactions were performed as described above for 2 hours. (H) Summary of autoubiquitination activity of parkin deletions (top) and missense mutations mapped onto the parkin structure and color-coded by activity (bottom). See fig. S8 for details.

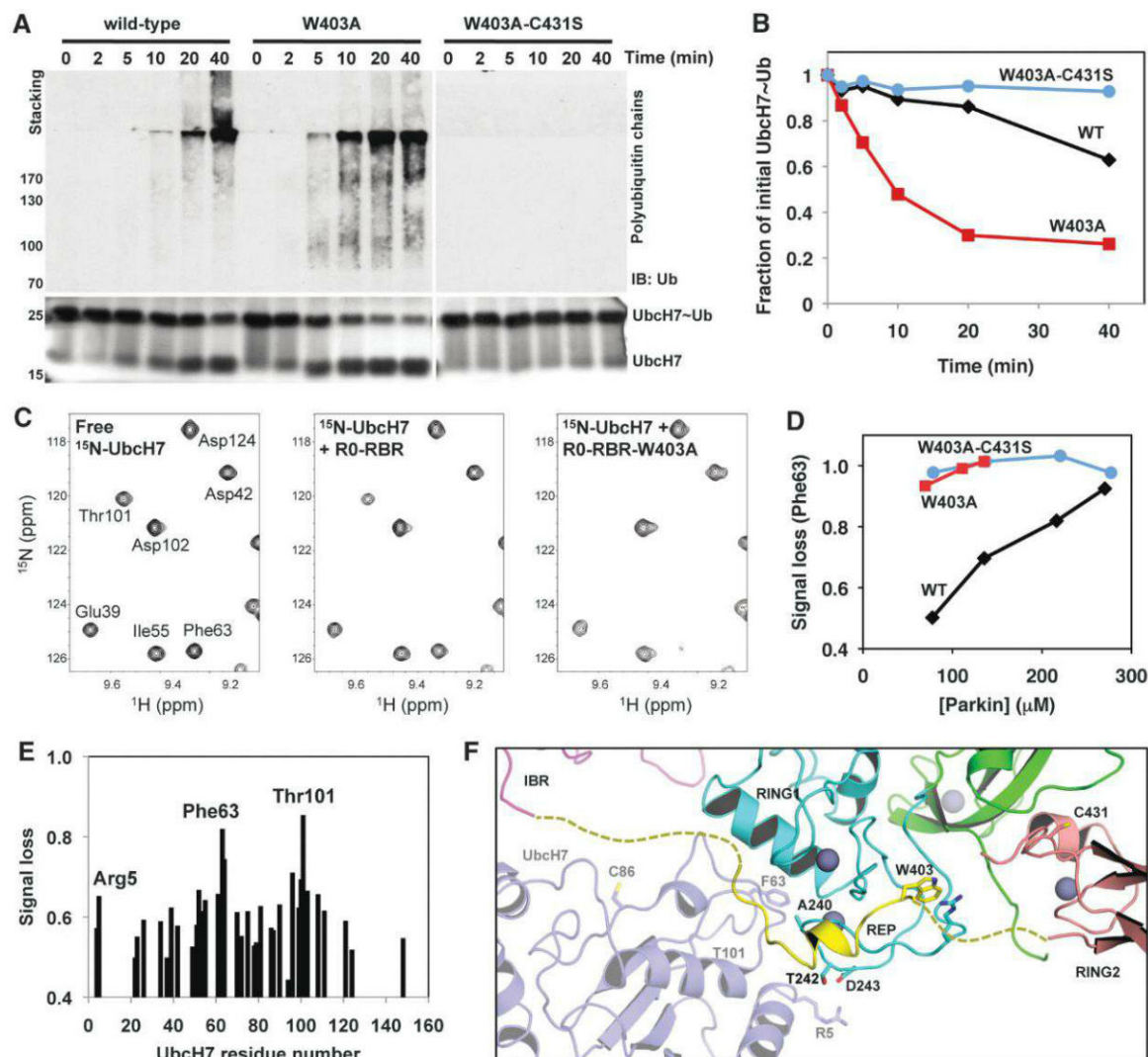


Fig. 3. Activation of parkin through increased UbchH7 binding. (A) UbchH7~Ub discharge assays with wild-type GST-parkin and mutants. Reactions were stopped with sample buffer containing tris(2-carboxyethyl)phosphine (TCEP) to reduce disulfide bonds but keep thioester bonds intact. Products were visualized using immunoblotting for polyubiquitin chains and silver staining for UbchH7. See fig. S10A for details. (B) Densitometric quantification of UbchH7~Ub discharging. Similar results were obtained under varying conditions (fig. S10, B and C). (C) NMR analysis of parkin binding to ¹⁵N-labeled UbchH7. Changes in the HSQC spectra of UbchH7 (150 μM) were caused by the addition of wild-type

R0-RBR (70 μM) or wild-type R0-RBR-W403A (70 μM). Peaks that undergo the most broadening (signal loss) correspond to the RING-binding site in UbchH7. (D) Quantification of amide NMR signal loss for Phe⁶³ as a function of different parkin construct concentrations. Spectra were normalized for dilution; plots show the number of scans and the peak intensity lost relative to the free UbchH7 spectrum. (E) Quantification of amide NMR signal loss for UbchH7 resonances after addition of 216 μM R0-RBR parkin. (F) Model of parkin bound to UbchH7. The REP occludes the UbchH7-binding site on RING1. Note the long distance between the active-site cysteines in UbchH7 (Cys⁸⁶) and parkin (Cys⁴³¹).

role in repressing parkin ligase activity through their interactions with RING1 and RING2, respectively (Fig. 2H).

We then asked whether the REP regulated parkin activity by affecting E2 binding to RING1. We used a ubiquitin-loaded E2 (E2~Ub) discharging assay to measure the rate of ubiquitin transfer from the E2 enzyme UbchH7. Wild-type parkin was moderately efficient at removing ubiquitin from UbchH7, concurrent with the slow formation of high-molecular weight polyubiquitinated protein (Fig. 3, A and B, and fig. S10). As expected, the W403A mutation increased the UbchH7~Ub discharging activity and polyubiquitin chain formation. The direct effect of the W403A mutant on E2 binding was measured using NMR spectroscopy.

Full-length and R0-RBR parkin bound UbchH7 weakly and led to line broadening and signal loss from UbchH7 residues at the E2-RING1 interface (Fig. 3, C to F, and fig. S11). Mutation of Trp⁴⁰³ in the REP strongly increased binding and led to complete loss of signals from the interfacial residues. The double W403A-C431S (Cys⁴³¹ → Ser) mutant also strongly bound UbchH7 but was completely impaired in UbchH7~Ub discharging (Fig. 3, A and B), which suggests that UbchH7 binding is upstream of Cys⁴³¹-mediated catalysis of ubiquitination.

We next addressed the role of the REP in the regulation of parkin activity in vivo. We expressed green fluorescent protein (GFP)-tagged parkin in HeLa cells and used time-lapse microscopy to

examine the kinetics of wild-type and mutant GFP-parkin recruitment to mitochondria after their depolarization with carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a proton ionophore (22). Recruitment of wild-type parkin to mitochondria began 30 min after CCCP treatment, with half of the cells showing recruitment at 45 min, whereas no recruitment of the catalytically inactive mutant was observed during the first hour (Fig. 4, A to C, fig. S12, and movies S1 and S2) as previously reported (22, 23). In contrast, the W403A mutant was recruited earlier than wild-type parkin with a lead time of ~10 min, implying that releasing autoinhibition by the REP predisposes parkin for mitochondrial recruitment. Nonetheless, recruitment of the W403A mutant to mitochondria was

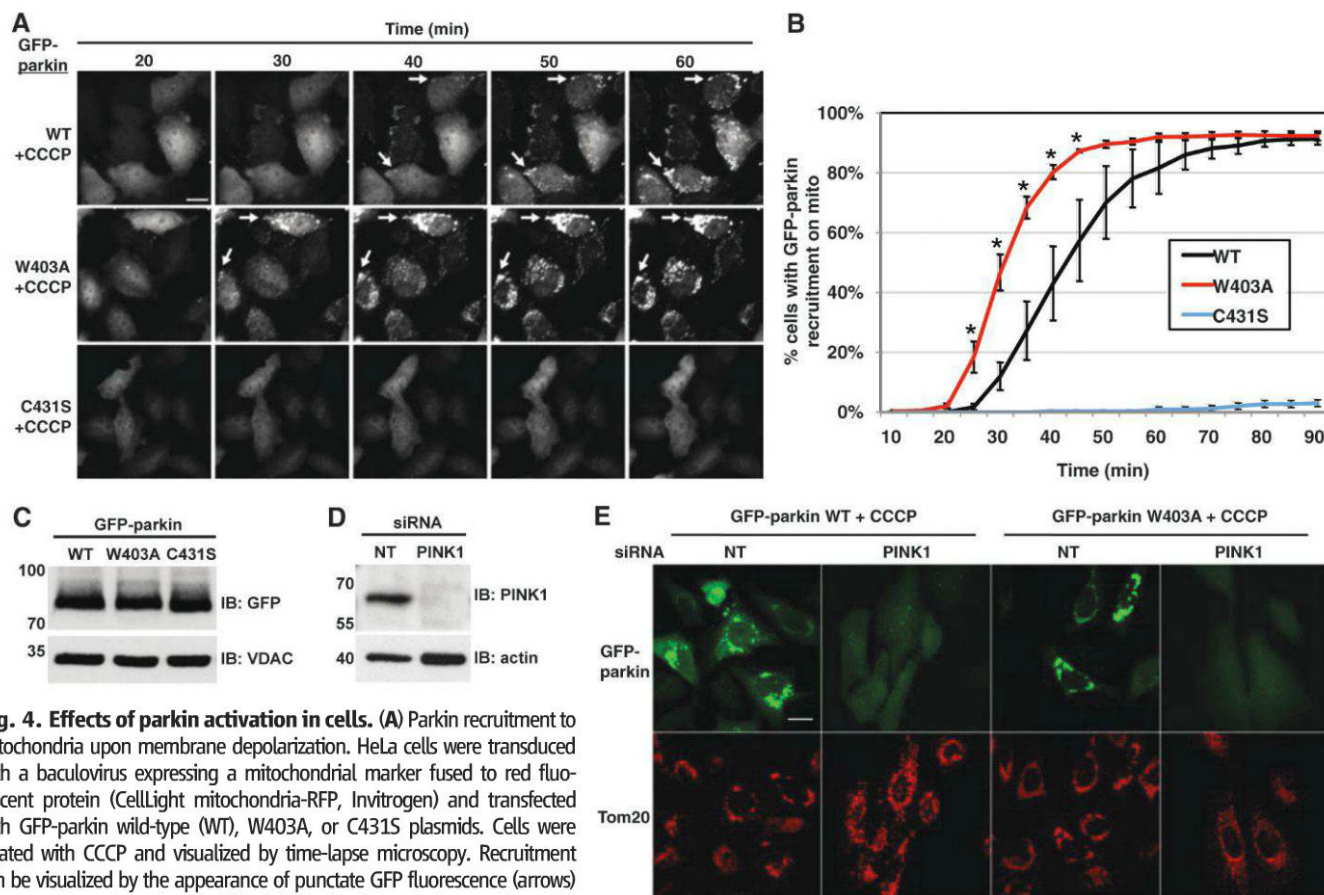


Fig. 4. Effects of parkin activation in cells. (A) Parkin recruitment to mitochondria upon membrane depolarization. HeLa cells were transduced with a baculovirus expressing a mitochondrial marker fused to red fluorescent protein (CellLight mitochondria-RFP, Invitrogen) and transfected with GFP-parkin wild-type (WT), W403A, or C431S plasmids. Cells were treated with CCCP and visualized by time-lapse microscopy. Recruitment can be visualized by the appearance of punctate GFP fluorescence (arrows) superimposed on the mitochondrial RFP (omitted for clarity; see fig. S12). Scale bar, 20 μ m. (B) Quantification of GFP-parkin recruitment to mitochondria. The percentage of cells showing recruitment of GFP-parkin to mitochondria was determined every 5 min. Error bars denote SEM ($N = 4$). * $P < 0.05$ (Student t test). (C) HeLa cells were transfected with GFP-parkin WT, W403A, or C431S plasmids for 24 hours. Whole-cell lysate proteins (20 μ g) were immunoblotted for GFP and voltage-dependent anion channel (VDAC). (D) HeLa cells were transfected with nontargeting

(NT) or PINK1 small interfering RNAs (siRNAs) for 24 hours and treated with CCCP for 3 hours. Whole-cell lysate proteins were immunoblotted for PINK1 and actin. (E) Parkin W403A recruitment to mitochondria is PINK1-dependent. HeLa cells were transfected with NT or PINK1 siRNAs before transfection of GFP-parkin WT or W403A (green) plasmids. Cells were treated with 20 μ M CCCP for 3 hours and immunostained for Tom20 (red). Scale bar, 20 μ m.

still PINK1-dependent (Fig. 4, D and E), similar to wild-type parkin (10–12). Thus, parkin activation appears to be a multistep process involving relocalization and release of ubiquitination activity, which are likely regulated through phosphorylation (10, 24–26), cysteine modification (27, 28), and ligand binding to the Ubl domain (5, 17, 20).

Whereas many PD-linked mutations reduce or abolish parkin activity, our findings reveal that it is possible to derepress parkin activity both in vitro and in vivo. Because parkin is neuroprotective in a number of PD models (7), the structure-based mechanism of activation presented here provides a potential framework to enhance parkin activity for therapeutics in PD.

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Supplementary Materials

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GPR15-Mediated Homing Controls Immune Homeostasis in the Large Intestine Mucosa

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Lymphocyte homing, which contributes to inflammation, has been studied extensively in the small intestine, but there is little known about homing to the large intestine, the site most commonly affected in inflammatory bowel disease. GPR15, an orphan heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptor, controlled the specific homing of T cells, particularly FOXP3⁺ regulatory T cells (T_{regs}), to the large intestine lamina propria (LILP). GPR15 expression was modulated by gut microbiota and transforming growth factor- β 1, but not by retinoic acid. GPR15-deficient mice were prone to develop more severe large intestine inflammation, which was rescued by the transfer of GPR15-sufficient T_{regs}. Our findings thus describe a T cell-homing receptor for LILP and indicate that GPR15 plays a role in mucosal immune tolerance largely by regulating the influx of T_{regs}.

The microbiota of the human gut have coevolved with the host (1–3), and their coexistence is due in large part to an equilibrium established with the host immune system (4). In the gastrointestinal tract, the large intestine harbors significantly more microbiota than the small intestine (5) and contains higher frequencies of FOXP3⁺ regulatory T cells (T_{regs}) (6–8). Disruption of the equilibrium between the host immune system and microbiota can trigger inflammatory bowel disease (IBD) in mouse models and, in humans, likely contributes to Crohn's disease and ulcerative colitis (9), in which the large intestine is the primary site of inflammation. Although T cell responses have critical roles in IBDs (9), it remains unclear how T cells migrate to the large intestine (10–12). Retinoic acid (RA) regulates lymphocyte migration to the small intestine but not to the large intestine (10, 11), which indicates that there is a separate mechanism for this process.

Human GPR15 (also known as BOB) was originally cloned as a co-receptor for HIV or the simian immunodeficiency virus (13, 14). To study the physiological function of its murine ortholog, we made knock-in mice in which endogenous *Gpr15* was replaced with the sequence for green fluorescent protein (GFP) (fig. S1). In humans, *GPR15* mRNA is highly expressed in the colon, peripheral blood lymphocytes (PBLs), and spleen (13). Similarly, in mice, GFP expres-

sion was detected in gut tissues and lymphoid organs, where it was largely restricted to T cell receptor β -positive (TCR β ⁺) cells (fig. S2, A and B). T cells in the large intestine lamina propria (LILP) exhibited the highest percentage of GFP⁺ cells, whereas GPR15 expression was minimal in other immune system cells in the LILP (fig. S2, C to F). To determine the functional characteristics of GPR15⁺ cells, we analyzed the transcriptomes of GFP⁺ and GFP⁺ CD4⁺ T cells from the LILP by microarray (table S1). Many of the genes highly expressed in GFP⁺ cells, compared with GFP⁺ cells, were characteristic of FOXP3⁺ T_{regs} [*Foxp3* (15), *Eos* (16), *interleukin-10* (*Il10*) (17), and *Cd25* (18)] (table S1). We confirmed the

preferential expression of GPR15 in T_{regs} by analyzing Foxp3 reporter expression in *Gpr15*^{gfp/+} *Foxp3*^{ires-mrpf} mice (19) (Fig. 1A) and also by staining for FOXP3 protein (fig. S2, G and H). About 60 to 70% of LILP CD4⁺FOXP3⁺ cells expressed *Gpr15*, compared with only 7 to 20% of CD4⁺FOXP3⁺ cells, in mice of two different genetic backgrounds (Fig. 1A and fig. S2H).

We next determined whether disproportionate expression of GPR15 in T_{regs} could affect their presence in the gut. We observed a reduction in the T_{reg} percentage in the LILP but not in the small intestine lamina propria (SILP) or spleen of *Gpr15* knockout (KO) compared with *Gpr15* heterozygous (Het) mice (Fig. 1B and fig. S3A). Both thymus-derived and peripherally derived T_{regs} were equally affected (fig. S3B). In cell numbers, T_{regs}, CD8⁺ T cells, and double-negative (DN) T cells, all of which showed significant GPR15-GFP expression, were reduced in the LILP of *Gpr15* KO mice (fig. S3C). These populations were unaffected in the SILP (fig. S3D). There was a significant, but much smaller, reduction in CD4⁺FOXP3⁺ T cells (fig. S3C), such that there was an overall decrease in T_{reg} percentage among total CD4⁺ T cells in the LILP (Fig. 1B and fig. S3A).

We next examined T_{reg} frequency in the LILP during an antigen-specific T cell response. *Rag2*^{−/−}, OT-II TCR transgenic mice that were heterozygous or homozygous for the *Gpr15*^{gfp} allele were fed with chicken ovalbumin (OVA). Without antigen exposure, all T cells maintained a naïve phenotype (CD44^{lo}), and no T_{reg} or GFP⁺ T cells were observed (fig. S4A). After OVA exposure of heterozygous mice, there was a small influx in the LILP of GFP⁺ T cells (2 to 5%) (fig. S4A) that were enriched for FOXP3 expression (fig. S4B). There was a significant reduction in

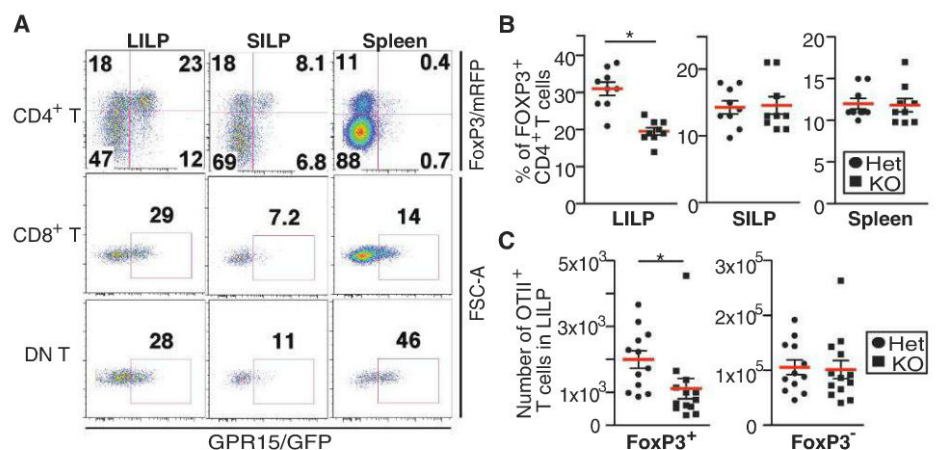


Fig. 1. GPR15 is preferentially expressed in and regulates the frequency of FOXP3⁺ regulatory T cells in the LILP. (A) *Gpr15*^{gfp/+} mice were bred to *Foxp3*^{ires-mrpf} mice. GFP and monomeric red fluorescent protein (mRFP) expression was examined in T cell subsets from different tissues (DN T: CD4[−]CD8 β [−] T cells). FSC-A, (forward-scatter). Results shown are representative of at least three independent experiments. (B) Percentage of FOXP3⁺ T_{regs} among CD4⁺ T cells in different tissues of *Gpr15*^{gfp/+} mice (Het) and *Gpr15*^{gfp/gfp} (KO) mice [B6N10 (C57BL/6-backcrossed 10 times): *n* = 9; combined from at least two independent experiments]. (C) Numbers of FOXP3⁺ (left) and FOXP3[−] cells (right) in the LILP were compared between OT-II *Rag2*^{−/−} *Gpr15*^{gfp/+} (Het) and OT-II *Rag2*^{−/−} *Gpr15*^{gfp/gfp} (KO) mice after OVA administration (*n* = 12, combined from four independent experiments). **P* < 0.05 (*t* test).

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the number and frequency of T_{regs} but not in the number of $CD4^+FOXP3^-$ T cells in the LILP of KO mice (Fig. 1C and fig. S4C). Thus, GPR15 preferentially contributes to T_{reg} frequency in the LILP at steady state and during an antigen-specific T cell response.

To determine whether GPR15 functions as a homing receptor for the LILP, we performed a short-term competitive homing assay by co-injecting T cells transduced with a control or a GPR15-encoding retrovirus into congenic hosts (fig. S5A). When GPR15⁺ cells and control cells were mixed at a 1:1 ratio and transferred into C57BL/6 mice, all tissues examined exhibited a 1:1 ratio of the donor-derived cells, except for the LILP, where there was a ~10-fold enrichment for GPR15⁺ cells (Fig. 2A and fig. S5B). There was minimal homing of transferred cells to the small intestine (fig. S5B). When GPR15⁺ cells were treated with the $G\alpha_i$ inhibitor pertussis toxin (PTX) before transfer, they were no longer enriched in the LILP (Fig. 2B), which indicated that GPR15 likely signals through $G\alpha_i$, as do other lymphocyte homing receptors. Many G protein-coupled receptors (GPCRs) have, in their second intracellular loop, a conserved DRY motif that is important for downstream signaling through its interactions with heterotrimeric G proteins (20). To ensure that active signaling through GPR15 was required for homing, we mutated the GPR15 DRY motif to DAY [Arg¹³¹ replaced by Ala (R131A)]. Although both wild-type (WT) and mutant proteins were similarly expressed at the cell surface (fig. S5C),

only cells expressing the WT fusion protein migrated to the LILP (Fig. 2C and fig. S5D).

Preferential homing of GPR15⁺ cells to the LILP was observed as early as 2 hours after cell transfer, which suggested that activation of this GPCR may promote integrin-dependent interaction of T cells with the endothelium in the target organ (fig. S5E). Indeed, blocking antibodies against either subunit of the $\alpha_4\beta_7$ integrin inhibited GPR15-mediated homing to the LILP (fig. S5F). Unlike $\alpha_4\beta_7$ and CCR9 (21), GPR15 was not induced by RA (fig. S6). However, GPR15 was induced in T cells treated with a combination of transforming growth factor- β 1 (TGF- β 1) and either IL-6 or IL-21 (fig. S7), and there was a marked decrease in *Gpr15* mRNA in T cells of *Tgfb1*^{C335/C335} mice that have reduced TGF- β 1 in vivo (22) (fig. S8, A and B). In contrast, *Il21r*^{-/-}*Il6*^{-/-} mice crossed with *Gpr15*^{gfp/+} mice had a similar level of GFP expression as control mice (fig. S8C), which suggested that only TGF- β 1 is a key regulator of GPR15 expression in vivo. Cells from *Gpr15* Het and KO mice were treated with these cytokines to induce GPR15 expression in vitro and were used in the short-term competitive homing assay (Fig. 2D). The results confirmed the importance of endogenously expressed GPR15 in the homing of T cells to the LILP.

We also tested the effect of gut microbiota on GPR15-mediated homing of T cells to the LILP. Treatment of *Gpr15*^{gfp/+} mice with a combination of broad-spectrum antibiotics led to a decrease in GPR15 expression (fig. S9A). In contrast, GPR15-

overexpressing T cells preferentially migrated to the LILP even in germ-free or antibiotics-treated recipients (fig. S9, B and C). Therefore, microbiota can affect GPR15 expression but are unlikely to produce ligand(s) for GPR15.

Because GPR15 deficiency affected T_{reg} homing to the LILP, we next investigated its role in immune homeostasis in the large intestine. We first examined cytokine production by $CD4^+$ T cells in the large intestines of *Gpr15* Het and KO mice. At steady state, there was an increased proportion of interferon (IFN)- γ - and IL-17A-producing cells among total $CD4^+$ T cells in the LILP of *Gpr15* KO mice on a 129/B6 mixed background (fig. S10A) but not in C57BL/6-backcrossed mice. However, when C57BL/6-backcrossed mice were injected with CD40 antibody [which induces acute colitis in *Rag2*^{-/-} mice (23)], inflammatory cytokine expression in the large intestine (but not spleen) was higher in *Gpr15* KO mice than in littermate controls (fig. S10, B and C). We next tested the physiological consequences of this inflammatory phenotype in an infection-induced colitis model. When mice were infected with *Citrobacter rodentium*, the majority of WT mice resolved inflammation and survived. In contrast, most *Gpr15* KO mice suffered severe weight loss and died (Fig. 3, A and B). KO mice also exhibited increased inflammation, tissue damage, and inflammatory cytokine expression (Fig. 3, C and D, and fig. S10D), and T_{reg} numbers in the LILP were reduced compared with Het mice (fig. S10E). The large intestine and spleen of Het and KO mice had a similar pathogen load (fig. S10F), which indicated that GPR15 is not required for controlling the infection but rather for dampening the immune response in the large intestine.

To confirm that sensitivity to *Citrobacter* infection was due to a role of GPR15 in T_{regs} rather than other T cells, we infected lethally irradiated mice that received mixed bone marrow from *Foxp3*^{sf} and from either WT or *Gpr15* KO mice. In *Foxp3*^{sf} + *Gpr15* KO mixed chimeras, T_{regs} will develop only from *Gpr15* KO bone marrow and will thus lack GPR15 expression, whereas other T cells can develop from GPR15-sufficient *Foxp3*^{sf} bone marrow. Indeed, *Foxp3*^{sf} + *Gpr15* KO mixed chimeric mice exhibited more severe inflammation and tissue damage than did chimeras generated with WT bone marrow (Fig. 3, E and F), which indicated that GPR15 expression in T_{regs} is required to prevent severe colitis following *Citrobacter* infection. This phenotype was not due to a role of GPR15 in regulating T_{reg} function, as WT T_{regs} and KO T_{regs} suppressed naïve T cell proliferation equally well (fig. S11A). These results indicate that GPR15 is critical for preventing pathological inflammation in the large intestine during colitis, most likely by regulating T_{reg} homing.

We next used a noninfectious model of colitis to determine the role of GPR15 in suppressing local inflammation in vivo. CD40 stimulation in the absence of adaptive immunity induces innate immune cell-mediated colitis (23) that can be

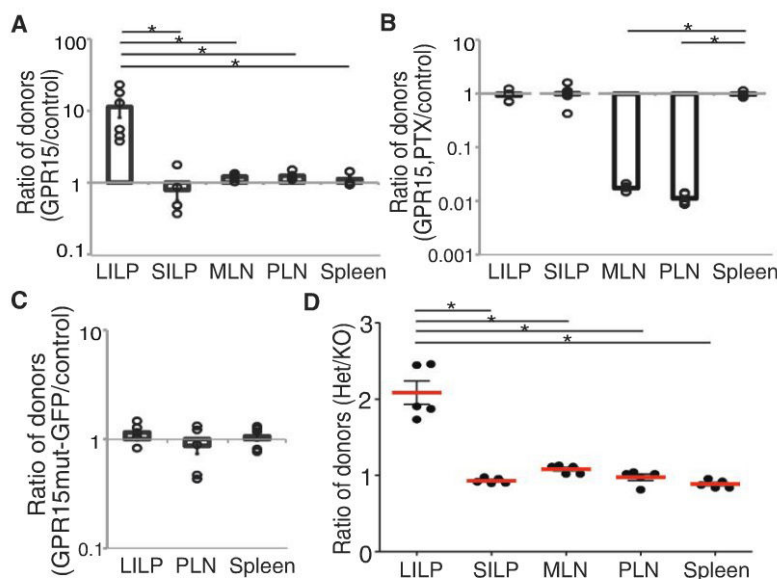


Fig. 2. GPR15 mediates T cell homing to the LILP. (A) Ratio of *Gpr15*-transduced and control-transduced donor cells in different tissues (MLN: mesenteric lymph nodes; PLN: inguinal, brachial, and axillary lymph nodes) 10 hours after transfer of an equal number of cells ($n = 6$, combined from three independent experiments). (B) Ratio of *Gpr15*-transduced cells treated with PTX and untreated control-transduced cells after cotransfer ($n = 5$). (C) Ratio of cells transduced with control vector and the R131A mutant *Gpr15* fused with *gfp* (GPR15mut-GFP) ($n = 7$, combined from three independent experiments). (D) Ratio in different tissues of $CD4^+$ T cells from *Gpr15*^{gfp/+} (Het) and *Gpr15*^{gfp/gfp} (KO) mice after in vitro culture in GPR15-inducing conditions and transfer of equal numbers of cells into recipients ($n = 5$, combined from two independent experiments). * $P < 0.05$ (t test).

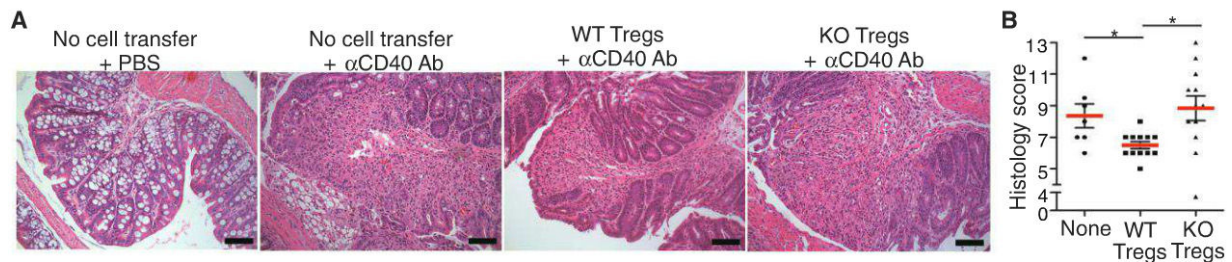
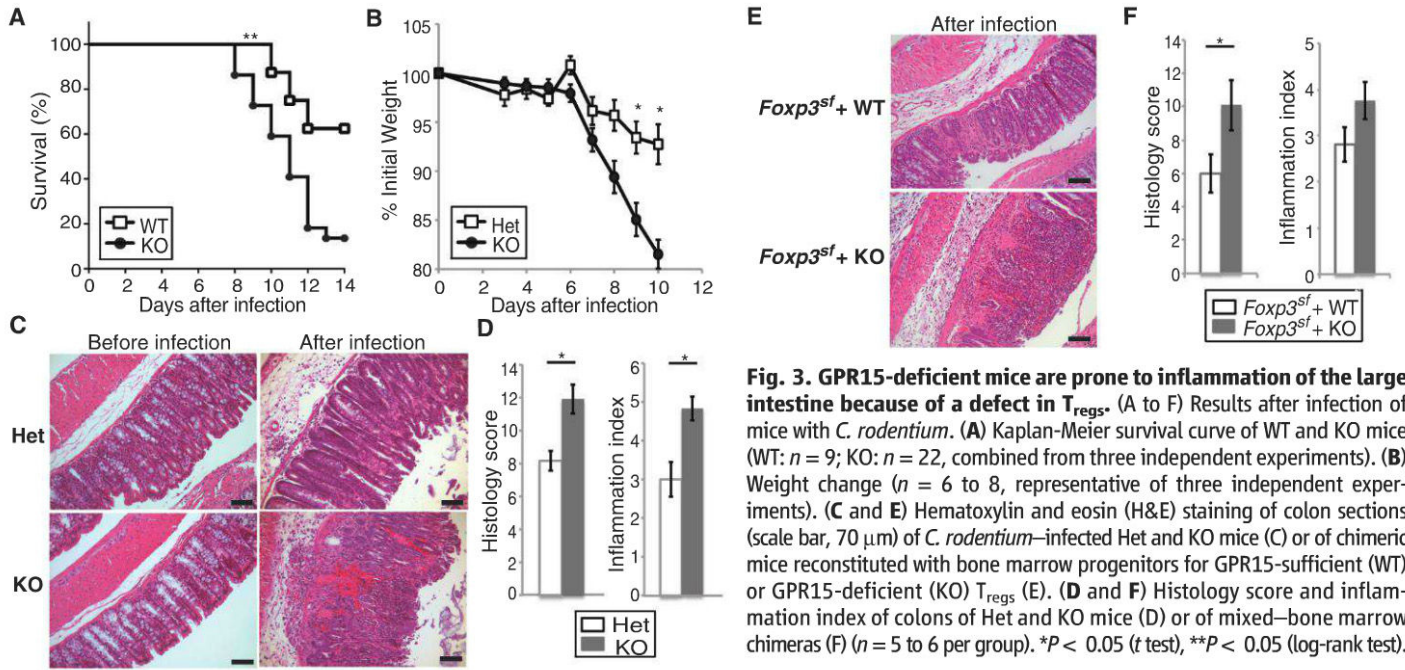


Fig. 4. T_{reg} s from GPR15-deficient mice cannot rescue colitis. (A and B) *Rag2*^{-/-} mice received 5×10^5 mRFP⁺ T_{reg} s transferred from either *Foxp3*^{ires-mrfp} or *Gpr15*^{glp/glp} *Foxp3*^{ires-mrfp} mice that were subsequently injected with CD40 antibody (FGK45). (A) H&E staining of proximal and midcolon

section of mice without any treatment, with colitis induction alone, or with colitis induction and rescue by WT or KO T_{reg} s (scale bar, $70 \mu\text{m}$). (B) Histology scores ($n = 7$ to 12 , combined from two independent experiments). * $P < 0.05$ (t test).

rescued by introduction of T_{reg} s (24, 25). We therefore transferred T_{reg} s from *Gpr15* WT or KO mice into *Rag2*^{-/-} mice that were subsequently treated with CD40 antibody. The transfer of WT T_{reg} s, but not KO T_{reg} s, reduced colitis severity and tissue damage (Fig. 4, A and B). We also determined the ability of naïve T cells from *Gpr15* WT and KO mice to induce colitis after *Helicobacter hepaticus* infection (24). In this T cell-transfer colitis model, which is dependent on the absence of T_{reg} s, KO $T_{naïve}$ cells induced colitis as well as their WT counterpart (fig. S11B), consistent with a preferential role for GPR15 in regulating the homing of T_{reg} s.

To determine whether the function of GPR15 is conserved between human and mouse, we examined *GPR15* mRNA expression in different cell types from various human tissues. Similar to *GPR15* expression in mice, that in humans was minimal in lymphocytes from the blood and the small intestine; however, it was expressed at high levels in lymphocytes from the large intestine (fig. S12). In contrast, we did not detect elevated

GPR15 mRNA expression in the T_{reg} -enriched CD25⁺CD4⁺ T cell population relative to other LILP T cell populations. Rather, there was more *GPR15* mRNA in CD25⁺CD4⁺ T cells than in CD25⁺CD4⁺ T cells. All colon samples were from colorectal carcinoma patients; thus, the pattern of GPR15 expression in colonic lymphocytes from normal subjects, IBD patients, and patients with HIV-mediated enteropathy (26, 27) remains to be determined.

Our results indicate that the small and the large intestine use different homing cues and different homing receptors for adaptive immune cells (fig. S13A) and, thereby, compartmentalize immune tolerance mediated by T_{reg} s (fig. S13B). GPR83 is also preferentially expressed in T_{reg} s and has been suggested to contribute to T_{reg} cell differentiation in inflammatory conditions (28). We cannot rule out a similar role for GPR15, in addition to its function in directing migration or retention of T cells in the large intestine. It will thus be interesting to examine the role of other inflammatory signals in GPR15-dependent T_{reg}

function. Our results improve understanding of immune homeostasis in the intestinal mucosa and could potentially lead to new therapeutic strategies to treat inflammatory diseases by combining in vitro expansion of T_{reg} s and GPR15 induction for reintroduction into patients.

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Supplementary Materials

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H5N1 Hybrid Viruses Bearing 2009/H1N1 Virus Genes Transmit in Guinea Pigs by Respiratory Droplet

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In the past, avian influenza viruses have crossed species barriers to trigger human pandemics by reassorting with mammal-infective viruses in intermediate livestock hosts. H5N1 viruses are able to infect pigs, and some of them have affinity for the mammalian type α -2,6-linked sialic acid airway receptor. Using reverse genetics, we systematically created 127 reassortant viruses between a duck isolate of H5N1, specifically retaining its hemagglutinin (HA) gene throughout, and a highly transmissible, human-infective H1N1 virus. We tested the virulence of the reassortants in mice as a correlate for virulence in humans and tested transmissibility in guinea pigs, which have both avian and mammalian types of airway receptor. Transmission studies showed that the H1N1 virus genes encoding acidic polymerase and nonstructural protein made the H5N1 virus transmissible by respiratory droplet between guinea pigs without killing them. Further experiments implicated other H1N1 genes in the enhancement of mammal-to-mammal transmission, including those that encode nucleoprotein, neuraminidase, and matrix, as well as mutations in H5 HA that improve affinity for humanlike airway receptors. Hence, avian H5N1 subtype viruses do have the potential to acquire mammalian transmissibility by reassortment in current agricultural scenarios.

Avian influenza viruses continue to evolve and spread, perpetuating the fear of an influenza pandemic if they acquire the ability to transmit efficiently among humans. The influenza virus genome comprises eight gene segments: basic polymerase 2 (PB2), basic polymerase 1 (PB1), acidic polymerase (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and nonstructural protein (NS). Hemagglutinin and neuraminidase are integral membrane proteins. The HA of human-infective

influenza subtypes preferentially recognizes α -2,6-linked sialic acids (SAs) (humanlike receptor), whereas the HA of avian-infective influenza subtypes preferentially recognizes α -2,3-linked SAs (avian-like receptor) (1). Combinations of amino acid changes such as 158D/224K/226L, 196R/226L/228S, or 110Y/160A/226L/228S (H3 numbering used throughout; see fig. S6) in HA protein can allow H5N1 viruses to recognize α -2,6-linked SAs, thereby conferring viral transmission between ferrets (2–4).

When two different influenza viruses infect the same cell, their genes can reassort to produce new viral strains. Historically, such reassortment has led to the emergence and spread of pandemic viruses in immunologically naïve human populations (5–8). A previous study with an H5N1 virus and a human H3N2 virus suggested that reassortments between these two subtypes to produce a

dangerous virus would be rare (9). However, both avian H5N1 and human 2009/H1N1 viruses have been found in pigs (10–14), so we asked: Could an H5N1 reassortant between avian H5N1 and the highly transmissible 2009/H1N1 virus become transmissible among mammals and potentially cause a human pandemic?

H5N1 influenza viruses were handled in the enhanced animal biosafety laboratory level 3 (ABSL3+) facility at the Harbin Veterinary Research Institute, China (15). All experimental studies with live H5N1 viruses were performed before the moratorium on such studies was in place (16, 17). Details of the biosafety and biosecurity measures taken and the dates on which the experiments were performed are provided in the supplementary materials.

We used two influenza viruses isolated in China: the H5N1 virus A/duck/Guangxi/35/2001 [DK/35(H5N1)] and the H1N1 virus A/Sichuan/1/2009 [SC/09(H1N1)]. DK/35(H5N1) is highly pathogenic for both chickens and mice (18). It transmits by direct contact among guinea pigs when they are housed together (19) but does not transmit between guinea pigs by respiratory droplet (Fig. 1A). We previously identified two molecular changes that are critical for the contact transmission of DK/35(H5N1) among guinea pigs: the asparagine residue at position 701 (701N) in PB2 and the alanine residue at position 160 (160A) in HA (19). The mutation of 160A, resulting in the absence of glycosylation at positions 158 to 160 in HA, permits virus binding to α -2,6-linked SAs (19, 20). Receptor specificity testing, using a solid-phase binding assay with four different glycans, indicated that DK/35(H5N1) binds to both α -2,3-linked SAs and α -2,6-linked SAs, and that its affinity to α -2,3-linked SAs is higher than to α -2,6-linked SAs (fig. S1A). SC/09(H1N1) was the first virus isolated in China during the 2009 influenza pandemic and transmits efficiently among guinea pigs by respiratory droplet (Fig. 1B) (21).

Using plasmid-based reverse genetics (22–24), we generated all possible reassortants possessing the H5 HA gene [i.e., 127 hybrid viruses between DK/35(H5N1) and SC/09(H1N1), 2⁷ minus one

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parent virus]. We numbered the gene segments derived from SC/09(H1N1) as follows: 1, *PB2*; 2, *PB1*; 3, *PA*; 5, *NP*; 6, *NA*; 7, *M*; and 8, *NS*. For example, “r123” denotes the reassortant containing SC/09(H1N1) *PB2*, *PB1*, and *PA*, with the other five segments from the DK/35(H5N1) virus. In previous studies (25, 26), certain H5 reassortants with H3N2 either were not generated or did not grow efficiently because of genomic incompatibility; for example, virus bearing the *NP* gene of human H3N2 virus and *M* and *NS* gene of H5N1 avian virus cannot replicate efficiently (25). By contrast, in this study, reassortants with the H5N1 *HA* were easily generated and all of them grew efficiently in chicken eggs, with viral titers ranging from 10^7 to 10^{10} 50% egg infectious doses (EID₅₀) (tables S1 to S3).

H5N1 virus virulence in mice correlates with its virulence in humans (27, 28), but this correlation has not been extended to ferrets (29). We used BALB/c mice to evaluate the virulence of our H5N1 reassortants by inoculating groups of eight mice with different doses of DK/35(H5N1) or SC/09(H1N1): DK/35(H5N1) had a mouse lethal dose (MLD₅₀; a dose lethal to 50% of mice) of $2.6 \log_{10}$ EID₅₀ (fig. S2A), whereas the MLD₅₀ of SC/09(H1N1) was $6.2 \log_{10}$ EID₅₀ (fig. S2B), indicating a milder infection. Virus replication was detected in the brains of mice that received 10^3 EID₅₀ or higher doses of DK/35(H5N1) but was detected in the brain of only one of three mice that were inoculated with 10^6 EID₅₀ of SC/09(H1N1) (fig. S2C). We did not measure the MLD₅₀ of all 127 viruses; instead, we infected mice with 10^3 EID₅₀ of each reassortant and scored them for replication in the brain and their effect on mouse mortality. On day 5 post-inoculation (p.i.), three mice in each group of eight were killed and their brain tissues were collected for virus titration; the other five mice were observed for a total of 14 days for body weight changes and death.

The 127 viruses were categorized into three groups according to their replication in the brain and lethality in mice: (i) 54 viruses showed similar pathogenicity to DK/35(H5N1); some viruses were lethal, but virus was not detected in the brains of all three mice. Mice infected with these viruses showed diversity in body weight changes at the end of the observation period (Fig. 2A, viruses shown in black, and table S1); (ii) 38 viruses were less pathogenic than DK/35(H5N1), they were not detected in the brain of any mouse killed on day 5 p.i., and the mice survived the infection and gained body weight (except those infected with the r23678 virus) (Fig. 2A, viruses shown in blue, and table S2); and (iii) 35 viruses were more pathogenic than DK/35(H5N1), they were detected in the brains of all three mice killed on day 5 p.i., all the remaining five mice died [mean time to death <10 days], and all lost 10 to 30% of their body weight (Fig. 2A, viruses shown in red, and table S3).

The activity of the influenza virus ribonucleoprotein (RNP) complex (i.e., the products of the

PB2, *PB1*, *PA*, and *NP* genes) is important for virus replication and virulence (30). The levels of RNP activity at 33° and 37°C correlate with viral replication efficiency in the upper and lower respiratory tracts, respectively (31, 32). One study suggests that replication efficiency of influenza virus in the upper respiratory tract favors viral transmission (31). Therefore, we tested virus RNA replication in human cells at both 33° and 37°C to determine the activities of the 16 combinations of RNP complex arising from DK/35(H5N1) and SC/09(H1N1). In general, these RNP complexes showed less activity at 33°C than at 37°C (Fig. 2B), except for two combinations that showed equivalent activity at both temperatures: (i) DK/35(H5N1) *PB2* and *NP* with SC/09(H1N1) *PB1* and *PA*, and (ii) DK/35(H5N1) *PB2*, *PB1*, and *NP* with SC/09(H1N1) *PA*. At 37°C, the RNP complex activity of wild-type DK/35(H5N1) was about 35% that of wild-type SC/09(H1N1) (Fig. 2B). All seven hybrid RNP combinations containing the SC/09(H1N1) *PA* were more active than the RNP of the parent SC/09(H1N1) at 37°C. All hybrid RNP combinations containing the SC/09(H1N1) *PB1* and the DK/35(H5N1) *PA* were less active than the RNP of wild-type DK/35(H5N1) at 37°C. Other combinations had intermediate activity.

A comparison of tables S1 to S3 with Fig. 2 reveals that 29 of the 32 viruses with an RNP containing the SC/09(H1N1) *PB1* and the DK/35(H5N1) *PA* were less pathogenic than DK/35(H5N1) (Fig. 2A, viruses shown in blue). All viruses with greater pathogenicity than DK/35(H5N1) contained an RNP that included the SC/09(H1N1) *PA* (Fig. 2A, viruses shown in red). The components of the RNP complex

therefore contribute to the virulence of the hybrid viruses in mice, and the combination of the *PA* gene of SC/09(H1N1) and the *PB1* gene of DK/35(H5N1) elevates the virulence of the reassortants.

We next determined whether any of these reassortants were transmissible in mammals. Guinea pigs and ferrets have been widely used as animal models for influenza virus transmission studies (2, 3, 19, 21, 33, 34), and human influenza viruses transmit similarly in these two models (21, 35, 36). Respiratory droplet transmission is restricted in ferrets if the virus does not exclusively or preferentially bind to α -2,6-linked SAs (37). Guinea pigs have both avian and mammalian types of airway receptor, but they did not support the respiratory droplet transmission of influenza virus that only binds to α -2,3-linked SAs (21). We used guinea pigs to test for respiratory droplet transmission of our H5N1 reassortants. We compared SC/09(H1N1) with DK/35(H5N1) plus reassortants r1, r12, r123, r125, r13, r135, r15, r2, r23, r235, r25, r3, r35, r5, and r1235 (the viruses listed in the first row of Fig. 2A). These H5N1 viruses contain different RNPs but have the same *HA*, *NA*, *M*, and *NS* genes of DK/35(H5N1). Two guinea pigs were intranasally (i.n.) inoculated with 10^6 EID₅₀ of each virus, and nasal washes and lung tissues from each guinea pig were collected on day 3 p.i. for viral titration. All viruses were detected in the nasal washes and lungs of these guinea pigs (Table 1).

Three guinea pigs were inoculated i.n. with 10^6 EID₅₀ of the test virus and housed in separate cages within an isolator. Twenty-four hours later, three naïve guinea pigs were placed in ad-

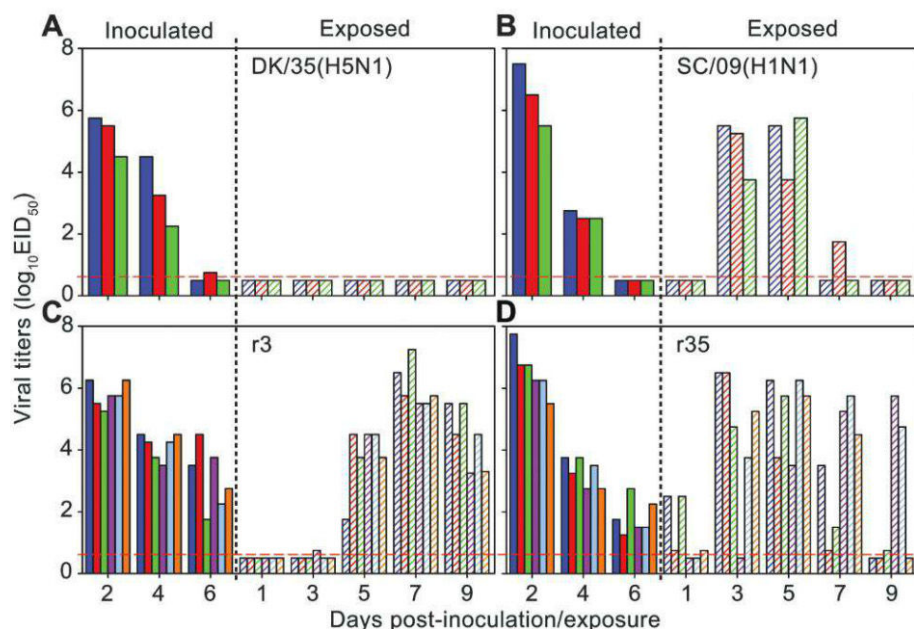


Fig. 1. Respiratory droplet transmission in guinea pigs of the DK/35 (H5N1) virus and its reassortants bearing different RNP complexes. (A) DK/35(H5N1); (B) SC/09(H1N1); (C) r3; (D) r35. Data for the 13 viruses that did not transmit are presented in fig. S3, A to M. Each color bar represents the virus titer from an individual animal. The dashed red lines indicate the lower limit of detection.

jacent cages. The six animals were separated by a double-layered net divider (4 cm apart) (fig. S3). Nasal washes were collected on days 2, 4, and 6 p.i. from the inoculated animals or on days 1, 3, 5, 7, and 9 post exposure (p.e.) from the exposed animals and titrated in eggs to test for respiratory droplet transmission. Sera were collected from all animals on day 21 p.i. for hemagglutinin inhibition antibody detection. Respiratory droplet transmission was confirmed when virus was detected in the nasal washes and by seroconversion of the naïve exposed animals at the end of the 3-week observation period.

Virus was detected in all directly infected animals (Fig. 1 and fig. S4) and in all three animals exposed to the guinea pigs that had been inoculated with SC/09(H1N1), r3 [containing the *PA* of SC/09(H1N1)], and r35 [containing the *PA* and *NP* of SC/09(H1N1)] (Fig. 1, B to D). Virus was detected later in the r3-exposed animals than in the r35-exposed animals, but no virus was detected in any animal exposed to those inoculated with DK/35(H5N1) (Fig. 1A) or the 13 hybrid viruses containing SC/09 *PB2* or *PB1* in their RNP complex (Table 1 and fig. S4). Six of these reassortants (r123, r1235, r13, r135, r23, and r235) had RNP complex activities comparable to or higher than those of SC/09(H1N1) at 33°C

(Fig. 2B). We therefore repeated the transmission experiments with these six viruses and the r3 and r35 reassortants and obtained the same results (the combined data from both experiments are shown in Fig. 1, C and D, and fig. S4, E to J). Of note, r35 was detected earlier than wild-type SC/09(H1N1) in the exposed animals (compare Fig. 1, B and D). Seroconversion occurred in the virus-inoculated animals and in all exposed animals that were virus-positive (Table 1). None of the guinea pigs died. These results indicate that the *PA* gene of SC/09(H1N1) alone can make DK/35(H5N1) transmissible by respiratory droplet between guinea pigs. Furthermore, the addition of the *NP* of SC/09(H1N1) appears to promote the transmission of r3 by respiratory droplet, although the *NP* of SC/09(H1N1) alone did not support virus transmission. Viruses bearing the *PB2* or *PB1* of SC/09(H1N1) in their RNP complexes were not transmissible, even though some also possessed the *PA* of SC/09(H1N1).

We also investigated the contributions of the nonpolymerase genes of SC/09(H1N1) to transmission by testing r678 and r1235678. Both viruses replicated well in the nasal cavities and lungs of guinea pigs (Table 1), and r1235678 and r678 were transmitted by respiratory droplet to two and three naïve animals, respectively (Fig. 3, A and B).

The contributions of the individual *NA*, *M*, and *NS* genes of SC/09(H1N1) to transmissibility were tested using r6, r7, and r8. Respiratory droplet transmission occurred in one of the three naïve animals for the r6 and r7 groups (Fig. 3, C and D). However, r8 transmitted to all three naïve animals by respiratory droplet. The *NA* and *M* genes are important for the highly transmissible phenotype of the 2009/H1N1 virus (38–40), but the *NS* gene was not previously known to function in influenza virus transmission. We therefore repeated the transmission experiment with r8 and found that the results were reproducible (the combined data from both experiments are shown in Fig. 3E).

Thus, both the *PA* and *NS* genes of SC/09(H1N1) can make DK/35(H5N1) highly transmissible by respiratory droplet between guinea pigs, and the *NA* and *M* of SC/09(H1N1) also promote H5N1 virus transmission through respiratory droplet. Moreover, the *NP* of SC/09(H1N1) accelerates H5N1 virus transmission when combined with the *PA* of SC/09(H1N1). Because r3678 transmitted with high efficiency in guinea pigs (Fig. 3F), other reassortants lethal to mice may also be transmissible by respiratory droplet between guinea pigs. Four viruses (r35, r678, r8, and r3678) were detectable in the naïve animals by day 1 p.e., indicating that these viruses

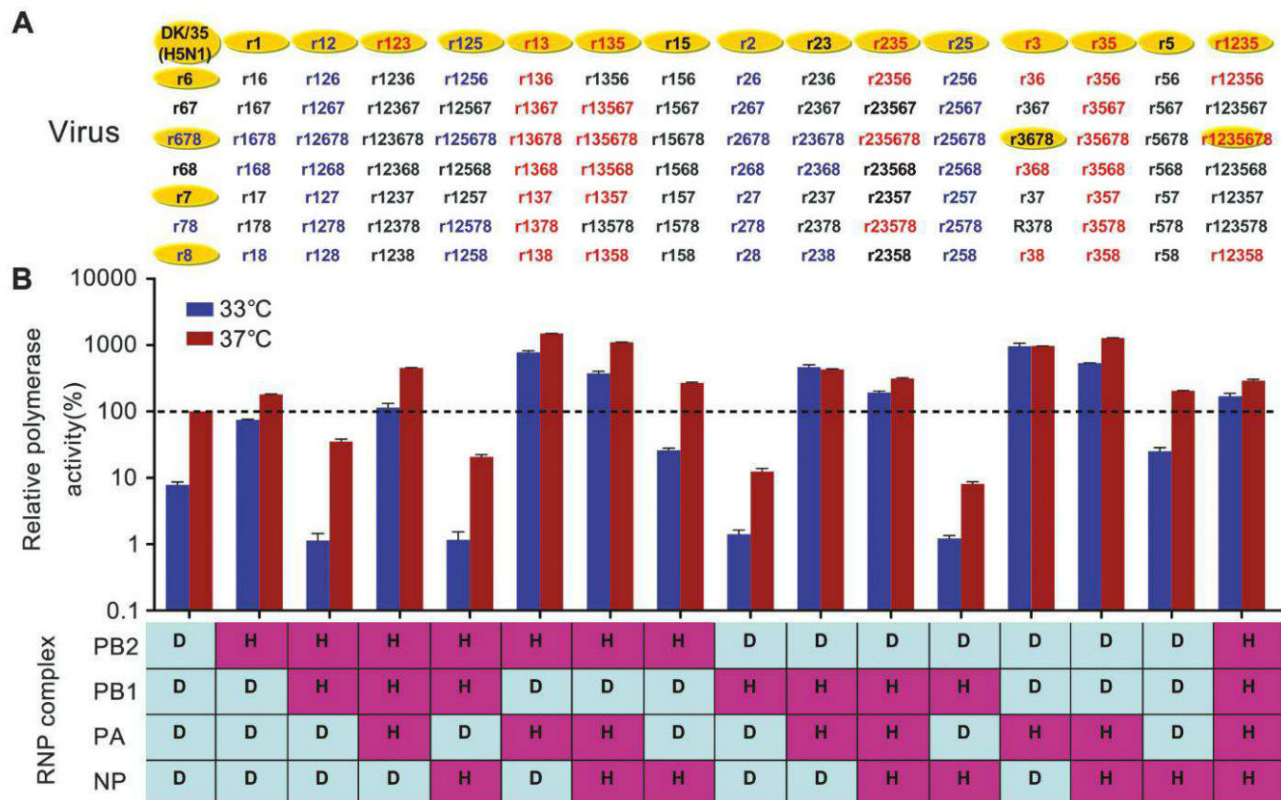


Fig. 2. Correlation of the virulence and the polymerase activity of H5N1 reassortants. (A) Pathogenicity of DK/35(H5N1) and SC/09(H1N1) reassortants. The colors of the viruses reflect their virulence in mice relative to DK/35(H5N1) as reported in tables S1 to S3 [red, more pathogenic than DK/35(H5N1); blue, less pathogenic than DK/35(H5N1); black, similar to DK/35(H5N1)]. Viruses highlighted in yellow were tested for respiratory droplet transmission in guinea pigs. (B) Polymerase activity of the 16 RNP

combinations of DK/35(H5N1) and SC/09(H1N1) viruses. The assay was performed at 37°C (red bars) or 33°C (blue bars). Values shown are means $\pm$ SD of three independent experiments and are standardized to those of DK/35(H5N1) measured at 37°C (100%, indicated by the dashed line). The chart below shows the parent subtype origin of the RNP complex. Segments derived from DK/35(H5N1) and those derived from SC/09(H1N1) are denoted D and H, respectively.

transmitted faster than the parent SC/09(H1N1), which was detected by day 3 p.e. (Fig. 1B). These transmission studies indicate that many of the

H5N1 hybrid viruses bearing one or more of the PA, NA, M, or NS genes of 2009/H1N1 were transmissible in guinea pigs.

Mutations in HA that confer exclusive binding to α -2,3-linked SAs eliminate the respiratory droplet transmission of 1918/H1N1 virus among

Table 1. Replication and seroconversion of guinea pigs inoculated with or exposed to the H5N1 virus DK/35 (H5N1) and its reassortants. In the virus name, “r” denotes reassortant. The numbers in the virus name indicate segments derived from the SC/09(H1N1) virus as follows: 1, PB2; 2, PB1; 3, PA; 5, NP; 6, NA; 7, M; and 8, NS. The virus segments derived from the DK/35(H5N1)

virus were not assigned numbers. Gene segments derived from DK/35(H5N1) and those derived from SC/09(H1N1) are denoted D and H, respectively. Viral titers are shown as individual titers for both guinea pigs. Sera were collected from the animals 3 weeks after virus inoculation or exposure; these animals were used for the transmission studies shown in Figs. 2 and 3. HI, hemagglutinin inhibition.

Virus name	Genotype								Viral titers (log ₁₀ EID ₅₀)		Seroconversion (HI titers), positive/total		Respiratory droplet transmission
	PB2	PB1	PA	HA	NP	NA	M	NS	Nasal	Lung	Inoculated	Exposed	
DK/35 (H5N1)	D	D	D	D	D	D	D	D	4.8, 3.5	2.8, 2.8	3/3 (40–80)	0/3	None
SC/09 (H1N1)	H	H	H	H	H	H	H	H	4.9, 5.2	3.3, 3.3	3/3 (320–1280)	3/3 (640)	Highly efficient
r1	H	D	D	D	D	D	D	D	3.5, 3.3	2.3, 3.3	3/3 (20–80)	0/3	None
r12	H	H	D	D	D	D	D	D	4.5, 2.5	1.8, 3.8	3/3 (20–40)	0/3	None
r123*	H	H	H	D	D	D	D	D	4.8, 5.3	4.8, 3.5	6/6 (40–80)	0/6	None
r125	H	H	D	D	H	D	D	D	4.5, 3.3	2.3, 3.3	3/3 (40–80)	0/3	None
r13*	H	D	H	D	D	D	D	D	4.8, 5.5	3.3, 1.8	6/6 (40–80)	0/6	None
r135*	H	D	H	D	H	D	D	D	4.3, 4.3	3.3, 2.3	6/6 (20–80)	0/6	None
r15	H	D	D	D	H	D	D	D	2.3, 3.5	4.8, 2.5	3/3 (20–40)	0/3	None
r2	D	H	D	D	D	D	D	D	4.8, 4.5	2.8, 2.5	3/3 (40–80)	0/3	None
r23*	D	H	H	D	D	D	D	D	3.3, 4.5	1.8, 2.8	6/6 (20–80)	0/6	None
r235*	D	H	H	D	H	D	D	D	4.3, 4.5	2.5, 2.8	6/6 (40–80)	0/6	None
r25	D	H	D	D	H	D	D	D	5.5, 5.3	4.3, 4.8	3/3 (20–40)	0/3	None
r3*	D	D	H	D	D	D	D	D	5.5, 4.8	2.8, 3.5	6/6 (80)	6/6 (20–40)	Highly efficient
r35*	D	D	H	D	H	D	D	D	4.3, 5.8	2.8, 2.8	6/6 (40–80)	6/6 (20–40)	Highly efficient
r5	D	D	D	D	H	D	D	D	5.5, 4.8	2.3, 1.3	3/3 (40–80)	0/3	None
r1235*	H	H	H	D	H	D	D	D	4.5, 4.3	2.5, 3.3	6/6 (20–80)	0/6	None
r1235678	H	H	H	D	H	H	H	H	4.8, 4.8	3.3, 2.8	3/3 (40–80)	2/3 (10–20)	Efficient
r678	D	D	D	D	D	H	H	H	4.3, 4.8	4.8, 3.5	3/3 (40–80)	3/3 (10–80)	Highly efficient
r6	D	D	D	D	D	H	D	D	4.3, 4.5	2.3, 3.8	3/3 (80)	1/3 (20)	Less efficient
r7	D	D	D	D	D	D	H	D	4.5, 5.3	4.5, 5.5	3/3 (40–80)	1/3 (40)	Less efficient
r8*	D	D	D	D	D	D	D	H	4.3, 4.8	4.5, 2.3	6/6 (20–160)	6/6 (20–80)	Highly efficient
r3678	D	D	H	D	D	H	H	H	5.5, 4.5	4.3, 3.8	3/3 (40–80)	3/3 (40–80)	Highly efficient

*Each transmission experiment was conducted twice, and the combined data from both experiments are presented in the table.

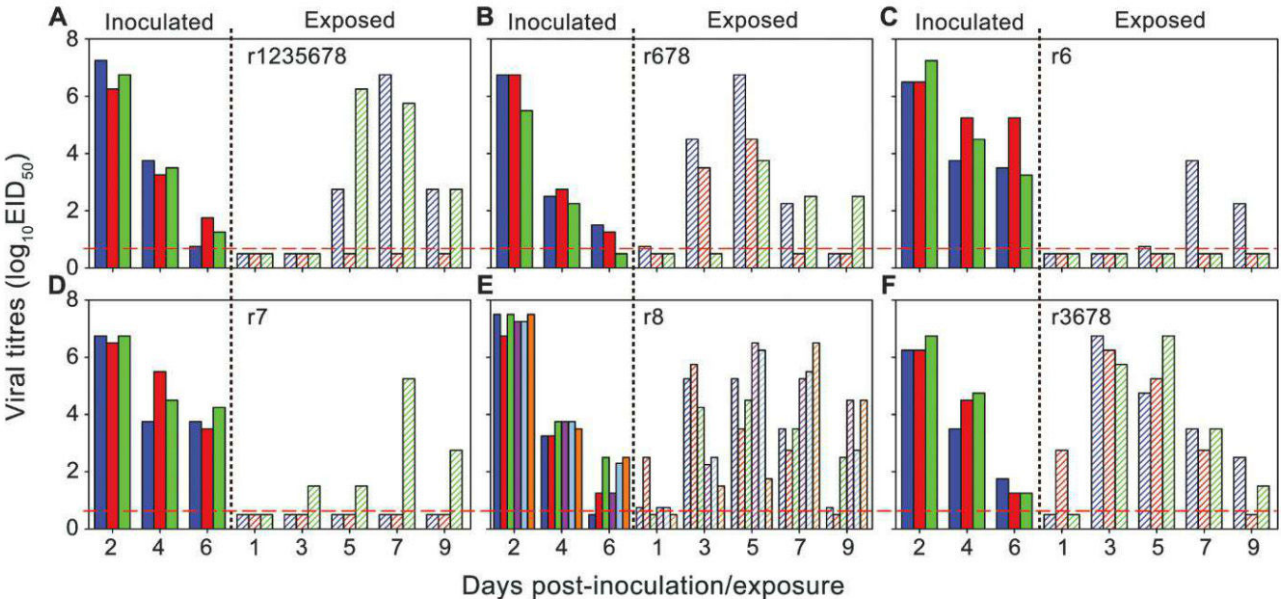


Fig. 3. Contributions of the NA, M, and NS genes of SC/09 (H1N1) to respiratory droplet transmission of H5N1 reassortants in guinea pigs. (A) r1235678; (B) r678; (C) r6; (D) r7; (E) r8 (data from two experiments); (F) r3678. Each color bar represents the virus titer from an individual animal. The dashed red lines indicate the lower limit of detection.

ferrets (37) and that of the 2009/H1N1 virus among both guinea pigs and ferrets (21), even if the other genes of these highly transmissible viruses are intact. We performed a solid-phase binding assay to investigate the receptor-binding specificity of the H5N1 viruses by using four different glycans (fig. S1). DK/35(H5N1), r3, r8, r35, r3678, and two H5N1 viruses isolated in nature—A/bar-headed goose/Qinghai/3/2005 and A/duck/Fujian/S4146/2010—bound to both α -2,3-linked SAs and α -2,6-linked SAs (fig. S1, A to G). A/duck/Anhui/1/2006 bound preferentially to α -2,3-linked SAs (fig. S1H). Although the affinity to α -2,6-linked SAs was lower than that to α -2,3-linked SAs, this property has been demonstrated to be required for the transmission of DK/35(H5N1) among guinea pigs by direct contact (19). It may therefore also be necessary for respiratory droplet transmission of H5N1 reassortants among guinea pigs.

We previously reported a DK/35(H5N1) mutant, DK/35(HA226L+228S), containing two amino acid changes at positions 226 and 228 in HA bound exclusively to α -2,6-linked SAs (19), and this property was further confirmed by the solid-phase binding assay (fig. S1I). DK/35(HA226L+228S), containing the combination of amino acids 160T/226L/228S that presented in the transmissible H5N1 virus reported by Herfst *et al.* (3), did not transmit between guinea pigs by respiratory droplet (fig. S4N), although very low virus titer was detected in preliminary experiments in one of three exposed ferrets (fig. S5F). We were unable to determine whether the gene combinations of the highly transmissible virus in guinea pigs would also make the DK/35(HA226L+228S) mutant transmissible in ferrets, because a moratorium on such studies was applied between 20 January 2012 and 20 January 2013 (16, 17).

Our studies provide evidence that H5N1 viruses that are capable of respiratory droplet transmission between mammals can be generated by reassortment between mammalian 2009/H1N1 and avian H5N1 viruses. Because the internal genes of these reassortants can already replicate efficiently in mammalian hosts, we predict that similar reassortants could infect humans and subsequently acquire mutations that improve binding efficacy for α -2,6-linked SAs. In fact, several combinations of amino acid changes of 158D, 160A, 224K, 226L, and 228S in HA allow the H5N1 virus to bind to α -2,6-linked SAs (2, 3, 19), and the mutations at 158D, 160A, and 224K have already been detected in H5N1 viruses circulating in nature (41).

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Supplementary Materials

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Materials and Methods
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An Airborne Transmissible Avian Influenza H5 Hemagglutinin Seen at the Atomic Level

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Recent studies have identified several mutations in the hemagglutinin (HA) protein that allow the highly pathogenic avian H5N1 influenza A virus to transmit between mammals by airborne route. Here, we determined the complex structures of wild-type and mutant HAs derived from an Indonesia H5N1 virus bound to either avian or human receptor sialic acid analogs. A cis/trans conformational change in the glycosidic linkage of the receptor analog was observed, which explains how the H5N1 virus alters its receptor-binding preference. Furthermore, the mutant HA possessed low affinities for both avian and human receptors. Our findings provide a structural and biophysical basis for the H5N1 adaptation to acquire human, but maintain avian, receptor-binding properties.

In the past 100 years, only three subtypes of influenza virus have adapted to human populations to cause four pandemics: H1N1 in 1918 and 2009, H2N2 in 1957, and H3N2 in 1968 (1–3). Other subtypes (e.g., H5N1, H6N1,

H7N2, and H9N2) have caused epizootics in domestic poultry in certain regions of the world (4), with a recent H7N9 human infection in China (5). H5N1 virus, especially, has spread through wild and domestic bird populations across Asia

and into Europe, the Middle East, and Africa (6, 7). H5N1 virus has also caused several hundred sporadic cases of human infections with high fatality (8–10), but it has not acquired the ability to efficiently transmit among humans.

Two recent studies identified several mutations that allow the H5N1 virus to become transmissible by airborne route in a ferret mammalian model system, raising the question of whether these mutations can also confer airborne transmissibility between humans (11, 12). Both reports show that several mutations in hemagglutinin

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(HA) of the H5N1 virus are sufficient to change the receptor-binding specificity from an avian receptor preference [α 2,3-linked sialic acid (SA) receptor] to a human receptor preference (α 2,6-linked SA receptor).

The mechanism by which the HAs of the H1, H2, and H3 subtypes bind to α 2,3- and α 2,6-linked SA receptors has been demonstrated previously (13), and studies have shown that for the HAs of the H2, H3, and H5 subtypes, two amino acid substitutions (Q226L and G228S) in the receptor binding site can switch the avian viruses to human-adapted viruses (14–16). In H1 subtype, the HA contains Q226 and G228, and the amino acids at positions 190 and 225 are important for the binding preference (17, 18). However, in H5 subtype, there is no structural evidence for how the receptor binding preference switches from avian to human receptors.

We generated soluble H5 HA protein (InH5) and its mutant (InH5mut, containing H110Y/T160A/Q226L/G228S; H3 numbering) from the influenza virus A/Indonesia/5/2005 to enable structural and binding studies (11). The genes encoding the HA ectodomains were cloned into the pFastbac1 vector (Invitrogen) and expressed by means of a baculovirus expression system (3). The recombinant proteins were purified with good purity and integrity (fig. S1) (19).

Surface plasmon resonance (SPR) experiments measured the binding affinities of InH5 and InH5mut to canonical avian and human receptors. The wild-type InH5 displayed a strong binding preference for the avian receptor, with an affinity of 60 μ M (Fig. 1, A and E), but weak binding to the human receptor (>1 mM, which is beyond the BIAcore measurement range) (Fig. 1, B and E). The InH5mut displayed significantly reduced binding affinity to the avian receptor (affinity of 554 μ M) (Fig. 1, C and F) and increased binding affinity to the human receptor (affinity of 372 μ M) (Fig. 1, D and F), changing the binding preference (Fig. 1, E and F). It is noteworthy that the InH5mut binding affinities for both the avian and human receptors were relatively low.

Using x-ray crystallography, we determined the structures of both InH5 and InH5mut to 2.5 and 2.9 Å, respectively. We solved the structures of both InH5 and InH5mut in complex with the two sialo-pentasaccharides LSTa and LSTc, which are natural sialosides from human milk (20). These sialo-pentasaccharides are analogs of the avian and human receptors, respectively, and contain the three terminal saccharides (Sia-Gal-GlcNAc) (20). There is unambiguous electron density for the ligands. (fig. S2).

Conventionally, the receptor binding site (RBS) of H5 is divided into two parts: the base, consisting of conserved residues Y98, W153 and H183; and the side, consisting of three secondary elements, i.e., the 130-loop, 190-helix, and 220-loop (13). The earlier crystal structures of H5 avian and H9 swine influenza bound to avian and human receptor analogs have identified the α 2,3-linkage-specific motif (trans conformation) in avian

H5 and the α 2,6-linkage-specific motif (cis conformation) in swine H9 (21).

The structure of InH5 with the avian receptor analog LSTa revealed that the ligand binds in a trans conformation (Fig. 2A), similar to that seen in a previously reported H5 HA-LSTa complex and nearly all other avian HA-LSTa complexes (13, 21). The “avian-signature” residue Q226 forms two hydrogen bonds with the ligand: one with the glycosidic oxygen of the α 2,3 linkage and the other with the 4-OH of Gal-2. Similarly, the structure of InH5 with the human receptor analog LSTc revealed that the ligand binds in a trans conformation (Fig. 2B), in contrast to all human HAs (which are observed in a cis con-

formation). In this structure, the “avian signature” residue Q226 makes three hydrogen bonds with Sia-1, with no hydrogen bonding to Gal-2 (table S1).

The structure of InH5mut with the avian receptor analog LSTa showed, in contrast to the wild-type complexes, that the ligand binds in a cis conformation (Fig. 2C). In this structure, the side-chain OH of S137 forms one hydrogen bond with Gal-2, which has not been observed in other HA-receptor complexes. The “human signature” residue L226 makes extremely weak van der Waals interactions (only two atom-to-atom contacts) with LSTa (table S1). In this case, most of the interactions are contributed by the 130-loop. Likewise, the structure of InH5mut with the human

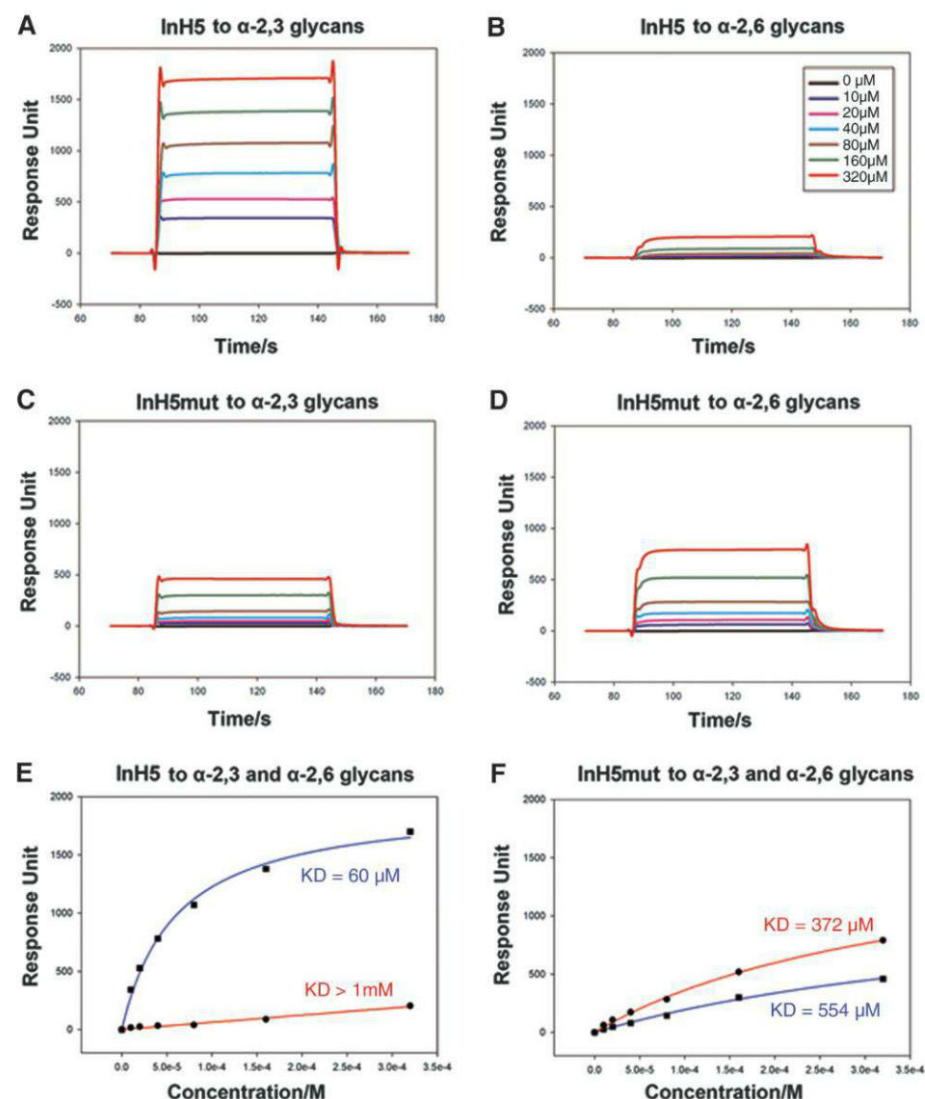


Fig. 1. BIAcore binding properties of the InH5 and InH5mut HAs to either α 2,3-linked or α 2,6-linked sialylglycan receptors. (A and B) BIAcore diagram of InH5 binding to the two receptors, showing strong binding to the α 2,3-linked sialylglycan receptor but little binding to the α 2,6-linked sialylglycan receptor. (C and D) BIAcore diagram of InH5mut binding to the two receptors, showing reduced binding to the α 2,3-linked sialylglycan receptor and increased binding to the α 2,6-linked sialylglycan receptor relative to InH5. (E and F) Response units were plotted against protein concentrations. Binding to α 2,3-linked sialylglycan receptor is colored in blue and α 2,6-linked sialylglycan receptor in red. The dissociation constant (K_D) values were calculated with a steady-state affinity model by the BIAcore 3000 analysis software (BIAevaluation Version 4.1).

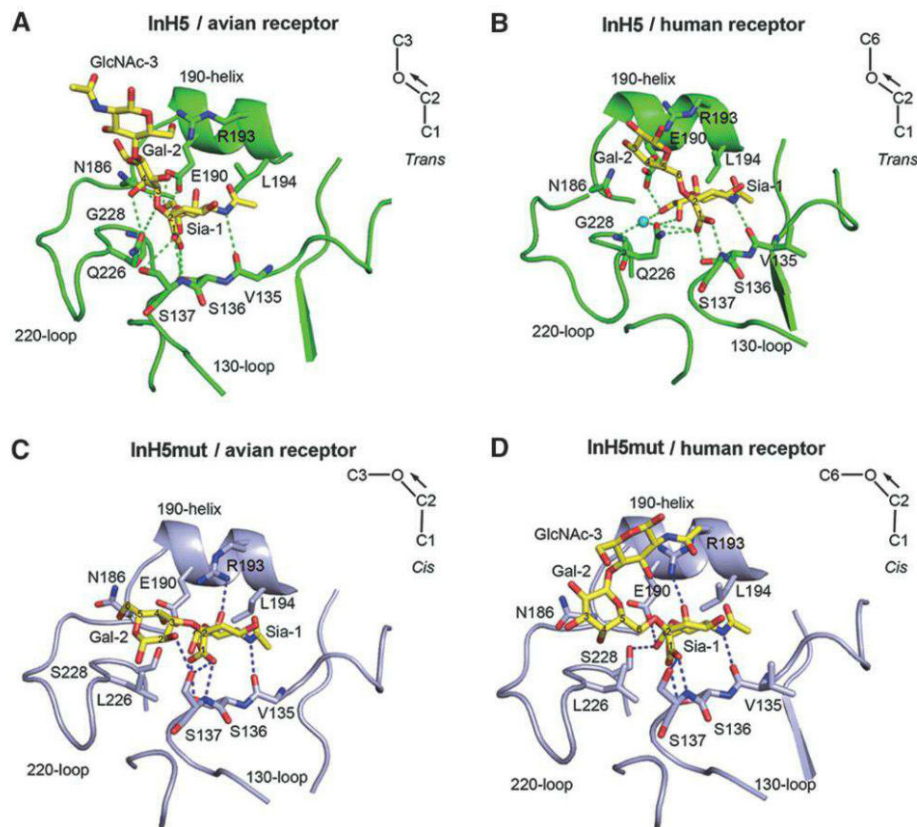


Fig. 2. Interaction of the InH5 and InH5mut HAs with either avian or human receptor analogs. The three secondary structural elements of the binding site (i.e., the 130-loop, 190-helix, and 220-loop) are labeled in ribbon representation, together with selected residues in stick representation. The hydrogen bonds are shown as dashed lines. The InH5 HA is colored in green, and the InH5mut HA is colored in light blue. The glycans are colored in yellow. (A and B) InH5 HA with the avian receptor analog LSTa ($\alpha 2,3$) pentasaccharide (A) or human receptor analog LSTc ($\alpha 2,6$) pentasaccharide (B) bound. Both LSTa and LSTc bind in a trans conformation. (C and D) InH5mut HA with the avian receptor analog LSTa (C) or the human receptor analog LSTc (D) bound. Both LSTa and LSTc bind in a cis conformation.

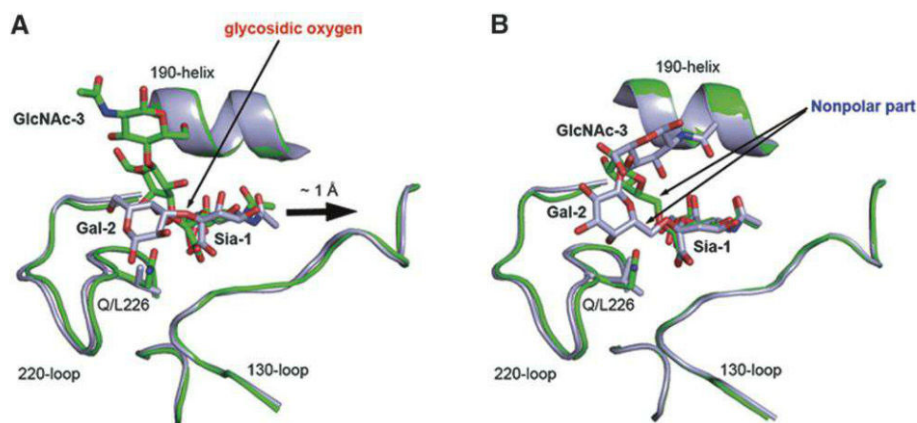


Fig. 3. Molecular mechanism of the cis/trans conformational switch when InH5 and InH5mut bind either avian or human receptors. (A) Comparison of the receptor binding sites between the InH5-LSTa (green) and InH5mut-LSTa (light blue) complexes. The sialic acid moves toward the 130-loop by ~ 1 Å in the InH5mut-LSTa complex structure relative to the InH5-LSTa complex structure. The hydrophilic glycosidic oxygen of LSTa is exposed to the hydrophilic residue Q226 in the InH5-LSTa complex, whereas the hydrophilic glycosidic oxygen is exposed away from the hydrophobic residue L226 in the InH5mut-LSTa complex. (B) Comparison of the receptor binding sites between the InH5-LSTc (green) and InH5mut-LSTc (light blue) complexes. The sialic acids are similarly located in both complexes. The nonpolar portion of LSTc is exposed to the hydrophobic residue L226 in the InH5mut-LSTc complex, whereas it is exposed away from the hydrophilic residue Q226 in the InH5-LSTc complex.

receptor analog LSTc showed that the ligand binds in a cis conformation (Fig. 2D). The “human signature” residue L226 makes stronger van der Waals interactions (eight atom-to-atom contacts) with LSTc than with LSTa (table S1).

Structural analysis indicates that a cis/trans conformational switch can occur when InH5 and InH5mut bind to different receptor analogs as a result of the Q226L substitution. The structures showed that in wild-type H5 HA bound to LSTa, the hydrophilic glycosidic oxygen became exposed to the hydrophilic residue Q226 (Fig. 3A), two hydrogen bonds bridged the Q226 to Sia-1 and Gal-2, and LSTa adopted a trans conformation. However, when the mutant HA was bound to LSTa, the hydrophobic residue L226 created an unfavorable environment for the hydrophilic glycosidic oxygen, and the glycosidic oxygen oriented away from L226, switching the conformation of LSTa from trans to cis and pushing the sialic acid receptor closer to the 130-loop by ~ 1 Å (Fig. 3A). This conformational switch may reduce the interaction between the InH5mut and LSTa.

By contrast, when wild-type HA binds to a mammalian receptor analog, LSTc, the hydrophilic residue Q226 creates an unfavorable environment for the nonpolar portion of LSTc and pushes it away from Q226 to adopt a trans conformation, which tilts the Gal-2 upward (Fig. 3B). However, when the mutant HA binds to LSTc, the hydrophobic residue L226 creates a favorable environment for the nonpolar portion of LSTc, and the nonpolar part orients toward the L226 to adopt a cis conformation (Fig. 3B) and makes a tighter interaction between the receptor and ligand. Hence, it appears that the avian and human receptors possess the inherent flexibility to accommodate different amino acid mutations in the RBS of HAs.

Previous studies demonstrated that RBSs of the human and swine influenza virus HAs are larger than those of the avian influenza virus HAs (21). We compared the wild-type H5 HA, the mutant, and the 1957 Singapore human H2 (57H2) to determine whether the Q226L and G228S substitutions alone can convert avian type HA into a human-like HA. The distance between the 130-loop and the 220-loop, which form two sides of the receptor binding site, is greater by ~ 1 Å in the InH5mut structure than in the InH5 structure (Fig. 4A). The distances between the 130-loop and 220-loop are comparable in the InH5mut and 57H2 structures (Fig. 4B). However, the mutant InH5mut does not display as strong a binding affinity for the human receptor as 57H2 does. Our structural comparisons showed a ~ 3 Å displacement in the InH5mut-LSTc complex relative to the 57H2-LSTc complex (Fig. 4C), resulting in fewer contacts with the receptor binding site. Further analysis revealed that InH5mut contains an arginine (R) at position 193 in the 190-helix whose long side chain might push the glycan away from the receptor binding site in the InH5mut-LSTc complex. By contrast, 57H2 has a threonine

(T) at the same position in its 190-helix whose side chain is much shorter than that of R193 (Fig. 4C). Similarly, the 1968 Hong Kong human H3 (68H3) contains an S193 in its 190-helix (Fig. 4D).

In summary, our binding studies *in vitro* showed that the wild-type HA of the avian H5N1 influenza virus protein preferentially binds to an analog of the avian receptor, whereas binding of the wild-type HA to a human sialic acid receptor analog was undetectable (>1 mM). If the H5 HA was mutated at Q226L, it acquired the ability to bind to both avian and human receptor analogs but with less affinity than that of wild-type HA binding to the avian receptor analog. Our structural studies showed that the mutation in HA at Q226L caused a trans/cis conformational switch in the glycan receptor that affected atomic contacts within the RBS and hence altered binding affinity. Our findings here might be expanded to explain the spread of the recent H7N9 virus (whose hemag-

glutinin has a natural Q226L substitution), which has a higher human infection rate in China (5).

In the HAs of the H2 and H3 subtypes, Q226L and G228S double-substitution mutations switch the receptor-binding properties from high affinity of avian receptor to high affinity of human receptor (22, 23). However, our SPR results demonstrated that InH5mut still has a low affinity for the human receptor, despite containing Q226L and G228S. The low affinity appears to be due to the long side chain of R193, which displaces the glycan from the receptor binding site by ~ 3 Å in the InH5mut-LSTc. This displacement decreased the atomic contacts between LSTc and the receptor binding site, resulting in a low binding affinity. Further substitutions may improve the affinity and thus transmissibility between humans.

Two other amino acid substitutions, H110Y and T160A, are also important for the transmissibility of avian H5 virus among mammals (fig.

S3A). Temperature-dependent circular dichroism (CD) spectroscopic experiments revealed that the thermostability of InH5mut is higher than that of wild-type InH5 proteins (fig. S3B), and structural comparison showed that Y110 in the InH5mut forms a hydrogen bond with the N413 of the adjacent monomer to stabilize the trimeric protein, whereas the H110 in the wild-type InH5 cannot do so (fig. S3, C and D). The T160A mutation, which results in the loss of a glycosylation site on the head of the HA close to the receptor binding site, enhances H5N1 virus binding to the $\alpha 2,6$ -linked human receptor (11, 12). However, in our InH5 structure, we did not observe the glycan in this glycosylation site owing to an artifact of baculovirus expression, and this structure will require further research. Moreover, this poor glycosylation in InH5 might explain the similar affinities of binding of mutant HA to human and avian receptor analogs, and the discrepancy with respect to the avian receptor binding of the mutant virus in studies by Herfst *et al.* (11) and Chutinimitkul *et al.* (24), who used different assays and substrates.

Other amino acid changes elsewhere in the virus may be critical to enable the H5N1 virus to transmit in humans. For example, Herfst *et al.* (11) introduced E627K into the PB2 protein (25, 26), together with the two substitutions introduced by reverse genetics and two acquired upon ferret passage in InH5mut, to generate a H5N1 virus that is transmissible among ferrets (11).

Our work therefore provides a structural basis to comprehensively evaluate the receptor binding properties of H5N1 virus.

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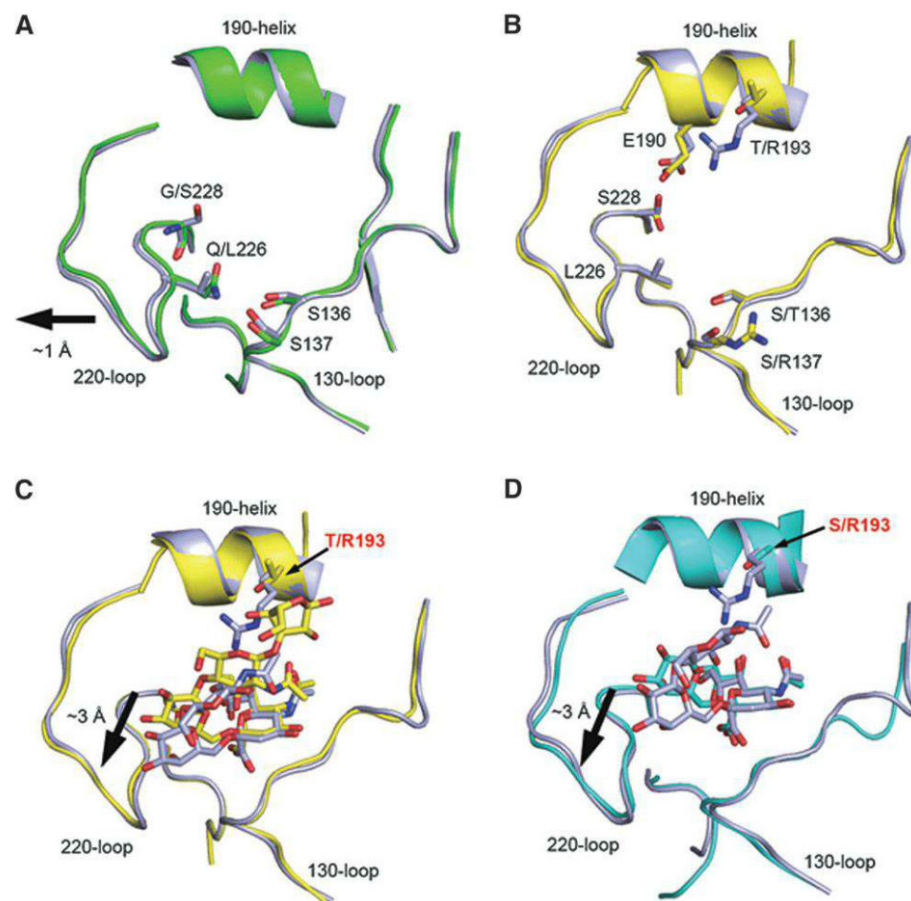


Fig. 4. Comparison of InH5, InH5mut, human H2/H3, and their HA-LSTc complex structures. (A) Comparison of receptor binding sites between InH5 (green) and InH5mut (light blue). The receptor binding site of InH5mut is ~ 1 Å wider than that of InH5 because of the clash between the hydrophobic residue L226 of the 220-loop and the hydrophilic residues S136 and S137 in the 130-loop. (B) Comparison of the receptor binding sites between InH5mut (light blue) and 57H2 (yellow). InH5mut has a similar wide receptor binding site compared to 57H2 (PDB code: 2WR7). (C and D) Comparison of the InH5mut-LSTc (light blue), human H2-LSTc (yellow), and human 68H3-LSTc (cyan) complexes (PDB code: 2YPG). The glycans are displaced away from the receptor binding site by ~ 3 Å in the InH5mut-LSTc complex relative to those in the 57H2-LSTc and 68H3-LSTc complexes. This displacement may result from the long side chain of R193, whereas the equivalent residues in human H2 and H3 (T and S) have short side chains.

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Supplementary Materials

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 Figs. S1 to S3
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GWAS of 126,559 Individuals Identifies Genetic Variants Associated with Educational Attainment

All authors with their affiliations appear at the end of this paper.

A genome-wide association study (GWAS) of educational attainment was conducted in a discovery sample of 101,069 individuals and a replication sample of 25,490. Three independent single-nucleotide polymorphisms (SNPs) are genome-wide significant (rs9320913, rs11584700, rs4851266), and all three replicate. Estimated effects sizes are small (coefficient of determination $R^2 \approx 0.02\%$), approximately 1 month of schooling per allele. A linear polygenic score from all measured SNPs accounts for $\approx 2\%$ of the variance in both educational attainment and cognitive function. Genes in the region of the loci have previously been associated with health, cognitive, and central nervous system phenotypes, and bioinformatics analyses suggest the involvement of the anterior caudate nucleus. These findings provide promising candidate SNPs for follow-up work, and our effect size estimates can anchor power analyses in social-science genetics.

Twin and family studies suggest that a broad range of psychological traits (1), economic preferences (2–4), and social and economic outcomes (5) are moderately heritable. Discovery of genetic variants associated with such traits may lead to insights regarding the biological pathways underlying human behavior. If the predictive power of a set of genetic variants considered jointly is sufficiently large, then a “risk score” that aggregates their effects could be useful to control for genetic factors that are otherwise unobserved, or to identify populations with certain genetic propensities, for example in the context of medical intervention (6).

To date, however, few if any robust associations between specific genetic variants and social-scientific outcomes have been identified probably because existing work [for a review, see (7)] has relied on samples that are too small [for discussion, see (4, 6, 8, 9)]. In this paper, we apply to a complex behavioral trait—educational attainment—an approach to gene discovery that has been successfully applied to medical and physical phenotypes (10), namely meta-analyzing data from multiple samples.

The phenotype of educational attainment is available in many samples with genotyped participants (5). Educational attainment is influenced by many known environmental factors, including public policies. Educational attainment is strongly associated with social outcomes, and there is a

well-documented health-education gradient (5, 11). Estimates suggest that around 40% of the variance in educational attainment is explained by genetic factors (5). Furthermore, educational attainment is moderately correlated with other heritable characteristics (1), including cognitive function (12) and personality traits related to persistence and self-discipline (13).

To create a harmonized measure of educational attainment, we coded study-specific measures using the International Standard Classification of Education (1997) scale (14). We analyzed a quantitative variable defined as an individual’s years of schooling (“EduYears”) and a binary variable for College completion (“College”). College may be more comparable across countries, whereas EduYears contains more information about individual differences within countries.

A genome-wide association study (GWAS) meta-analysis was performed across 42 cohorts in the discovery phase. The overall discovery sample comprises 101,069 individuals for EduYears and 95,427 for College. Analyses were performed at the cohort level according to a prespecified analysis plan, which restricted the sample to Caucasians (to help reduce stratification concerns). Educational attainment was measured at an age at which participants were very likely to have completed their education [more than 95% of the sample was at least 30 (5)]. On average, participants have 13.3 years of schooling, and 23.1% have a

College degree. To enable pooling of GWAS results, all studies conducted analyses with data imputed to the HapMap 2 CEU (r22.b36) reference set. To guard against population stratification, the first four principal components of the genotypic data were included as controls in all the cohort-level analyses. All study-specific GWAS results were quality controlled, cross-checked, and meta-analyzed using single genomic control and a sample-size weighting scheme at three independent analysis centers.

At the cohort level, there is little evidence of general inflation of P values. As in previous GWA studies of complex traits (15), the Q-Q plot of the meta-analysis exhibits strong inflation. This inflation is not driven by specific cohorts and is expected for a highly polygenic phenotype even in the absence of population stratification (16).

From the discovery stage, we identified one genome-wide-significant locus (rs9320913, $P = 4.2 \times 10^{-9}$) and three suggestive loci (defined as $P < 10^{-6}$) for EduYears. For College, we identified two genome-wide-significant loci (rs11584700, $P = 2.1 \times 10^{-9}$, and rs4851266, $P = 2.2 \times 10^{-9}$) and an additional four suggestive loci (Table 1). We conducted replication analyses in 12 additional, independent cohorts that became available after the completion of the discovery meta-analysis, using the same pre-specified analysis plan. For both EduYears and College, the replication sample comprises 25,490 individuals.

For each of the 10 loci that reached at least suggestive significance, we brought forward for replication the single-nucleotide polymorphism (SNP) with the lowest P value. The three genome-wide-significant SNPs replicate at the Bonferroni-adjusted 5% level, with point estimates of the same sign and similar magnitude (Fig. 1 and Table 1). The seven loci that did not reach genome-wide significance did not replicate (the effect went in the anticipated direction in five out of seven cases). The meta-analytic findings are not driven by extreme results in a small number of cohorts (see P_{het} in Table 1), by cohorts from a specific geographic region (figs. S7 to S15), or by a single sex (figs. S3 to S6). Given the high correlation between EduYears and College (5), it is unsurprising that the set of SNPs with low P values exhibit considerable overlap in the two analyses (tables S8 and S9).

The observed effect sizes of the three replicated individual SNPs are small [see (5) for discussion]. For EduYears, the strongest effect identified (rs9320913) explains 0.022% of phenotypic

variance in the replication sample. This coefficient of determination R^2 corresponds to a difference of ≈ 1 month of schooling per allele. For College completion, the SNP with the strongest estimated effect (rs11584700) has an odds ratio of 0.912 in the replication sample, equivalent to a 1.8 percentage-point difference per allele in the frequency of completing College.

We subsequently conducted a “combined stage” meta-analysis, including both the discovery and replication samples. This analysis revealed additional genome-wide-significant SNPs: four for EduYears and three for College. Three of these SNPs (rs1487441, rs11584700, rs4851264) are in linkage disequilibrium with the replicated SNPs. The remaining four are located in different loci and warrant replication attempts in future research: rs7309, a 3′ untranslated region (3′UTR) variant in *TANK*; rs11687170, close to *GBX2*; rs1056667, a 3′UTR variant in *BTN1A1*; and rs13401104 in *ASB18*.

Using the results of the combined meta-analyses of discovery and replication cohorts, we conducted a series of complementary and exploratory supplemental analyses to aid in interpreting and contextualizing the results: gene-based association tests, expression quantitative trait locus (eQTL) analyses of brain and blood tissue data, pathway analysis, functional annotation searches, enrichment analysis for cell-type-specific overlap with H3K4me3 chromatin marks, and predictions of likely gene function with the use of gene-expression data. Table S20 summarizes promising candidate loci identified through follow-up analyses (5). Two regions, in particular, showed convergent evidence from functional annotation, blood cis-eQTL analyses, and gene-based tests: chromosome 1q32 (including *LRRN2*, *MDM4*, and *PIK3C2B*) and chromosome 6 near the major histocompatibility complex. We also find evidence that in anterior caudate cells, there is enrichment of H3K4me3 chromatin marks (believed to be more common in active regulatory regions) in the genomic regions implicated by our analyses (fig. S20). Many of the implicated genes have previously been associated with health, central nervous system, or cognitive-process phenotypes in either human GWAS or model-animal studies (table S22). Gene coexpression analysis revealed that several implicated genes (including *BSN*, *GBX2*, *LRRN2*, and *PIK3C2B*) are probably involved in pathways related to cognitive processes (such as learning and long-term memory) and neuronal development or function (table S21).

Although the effects of individual SNPs on educational attainment are small, many of their potential uses in social science depend on their combined explanatory power. To evaluate the combined explanatory power, we constructed a linear polygenic score (5) for each of our two education measures using the meta-analysis results (combining discovery and replication), excluding one cohort. We tested these scores for association with educational attainment in the

Table 1. The results of the GWAS meta-analysis for the independent signals reaching $P < 10^{-6}$ in the discovery stage. The rows in bold are the independent signals reaching $P < 5 \times 10^{-8}$ in the discovery stage. “Frequency” refers to allele-frequency in the combined-stage meta-analysis. “Beta/OR” refers to the effect size in the EduYears analysis and to the odds ratio in the College analysis. All P values are from the sample-size-weighted meta-analysis (fixed effects). The P value in the replication-stage meta-analysis was calculated from a one-sided test. r^2 represents the percent heterogeneity of effect size between the discovery-stage studies. P_{het} is the heterogeneity P value. bp, base pair.

SNP	Chr	Position (bp)	Nearest gene	Reference allele	Discovery stage			Replication stage			Combined stage			Combined stage—sex-specific analysis				
					Beta/OR	P value	r ²	P _{het}	Beta/OR	P value	Beta/OR	P value	P _{het}	Beta/OR (males)	P value (males)	Beta/OR (females)	P value (females)	
EduYears																		
rs9320913	6	98691454	LOC100129158	A	0.483	4.19×10 ⁻⁹	18.3	0.097	0.077	0.012	0.101	3.50×10 ⁻¹⁰	0.350	0.095	1.87×10 ⁻⁴	0.100	1.43×10 ⁻⁶	
rs3783006	13	97909210	STK24	C	0.454	2.29×10 ⁻⁷	0	0.982	0.056	0.055	0.088	8.45×10 ⁻⁸	0.959	0.064	1.44×10 ⁻²	0.108	3.35×10 ⁻⁷	
rs8049439	16	28745016	ATXN2L	T	0.581	7.12×10 ⁻⁷	10.7	0.229	0.065	0.026	0.086	1.15×10 ⁻⁷	0.205	0.097	1.43×10 ⁻⁴	0.078	1.90×10 ⁻⁴	
rs13188378	5	101958587	SLC6A1	A	0.878	7.49×10 ⁻⁷	0	0.791	0.091	0.914	-0.097	1.37×10 ⁻⁴	0.646	-0.134	8.21×10 ⁻³	-0.080	5.92×10 ⁻³	
College																		
rs11584700	1	202843606	LRRN2	A	0.780	2.07×10 ⁻⁹	13.8	0.179	0.912	4.86×10 ⁻⁴	0.919	8.24×10 ⁻¹²	0.221	0.934	6.11×10 ⁻⁴	0.911	2.12×10 ⁻⁹	
rs4851266	2	100184911	LOC150577	T	0.396	2.20×10 ⁻⁹	23.7	0.049	1.049	0.003	1.050	5.33×10 ⁻¹¹	0.072	1.054	1.55×10 ⁻⁵	1.052	6.74×10 ⁻⁸	
rs2054125	2	199093966	PLCL1	T	0.064	1.468	5.55×10 ⁻⁸	7	0.325	1.098	0.225	1.376	2.12×10 ⁻⁷	0.268	1.264	1.74×10 ⁻²	1.503	1.95×10 ⁻⁷
rs3227	6	33770273	ITPR3	C	0.498	1.043	6.02×10 ⁻⁸	5	0.363	1.010	0.280	1.037	3.24×10 ⁻⁷	0.415	1.046	9.44×10 ⁻⁵	1.029	1.37×10 ⁻³
rs4073894	7	104254200	LHFPL3	A	0.207	1.076	4.41×10 ⁻⁷	0	0.765	1.003	0.467	1.062	5.55×10 ⁻⁶	0.513	1.050	2.18×10 ⁻²	1.073	1.74×10 ⁻⁵
rs12640626	4	176863266	GPM6A	A	0.580	1.041	4.94×10 ⁻⁷	10.9	0.234	1.000	0.495	1.034	7.48×10 ⁻⁶	0.420	1.038	1.59×10 ⁻³	1.031	7.61×10 ⁻⁴

excluded cohort. We constructed the scores using SNPs whose nominal P values fall below a certain threshold, ranging from 5×10^{-8} (only the genome-wide-significant SNPs were included) to 1 (all SNPs were included).

We replicated this procedure with two of the largest cohorts in the study, both of which are family-based samples [Queensland Institute of Medical Research (QIMR) and Swedish Twin Registry (STR)]. The results suggest that educational attainment is a highly polygenic trait (Fig. 2 and table S23): the amount of variance accounted for increases as the P value threshold becomes less conservative (i.e., includes more SNPs). The linear polygenic score from all measured SNPs accounts for $\approx 2\%$ ($P = 1.0 \times 10^{-29}$) of the variance in EduYears in the STR sample and $\approx 3\%$ ($P = 7.1 \times 10^{-24}$) in the QIMR sample.

To explore one of the many potential mediating endophenotypes, we examined how much the same polygenic scores (constructed to explain EduYears or College) could explain individual differences in cognitive function. Though it would have been preferable to explore a richer set of mediators, this variable was available in STR, a data set where we had access to the individual-level genotypic data. The Swedish Enlistment Battery (used for conscription) had

previously been administered to measure cognitive function in a subset of males (5, 17). The estimated $R^2 \approx 2.5\%$ ($P < 1.0 \times 10^{-8}$) for cognitive function is actually slightly larger than the fraction of variance in educational attainment captured by the score in the STR sample. One possible interpretation is that some of the SNPs used to construct the score matter for education through their stronger, more direct effects on cognitive function (5). A mediation analysis (table S24) provides tentative evidence consistent with this interpretation.

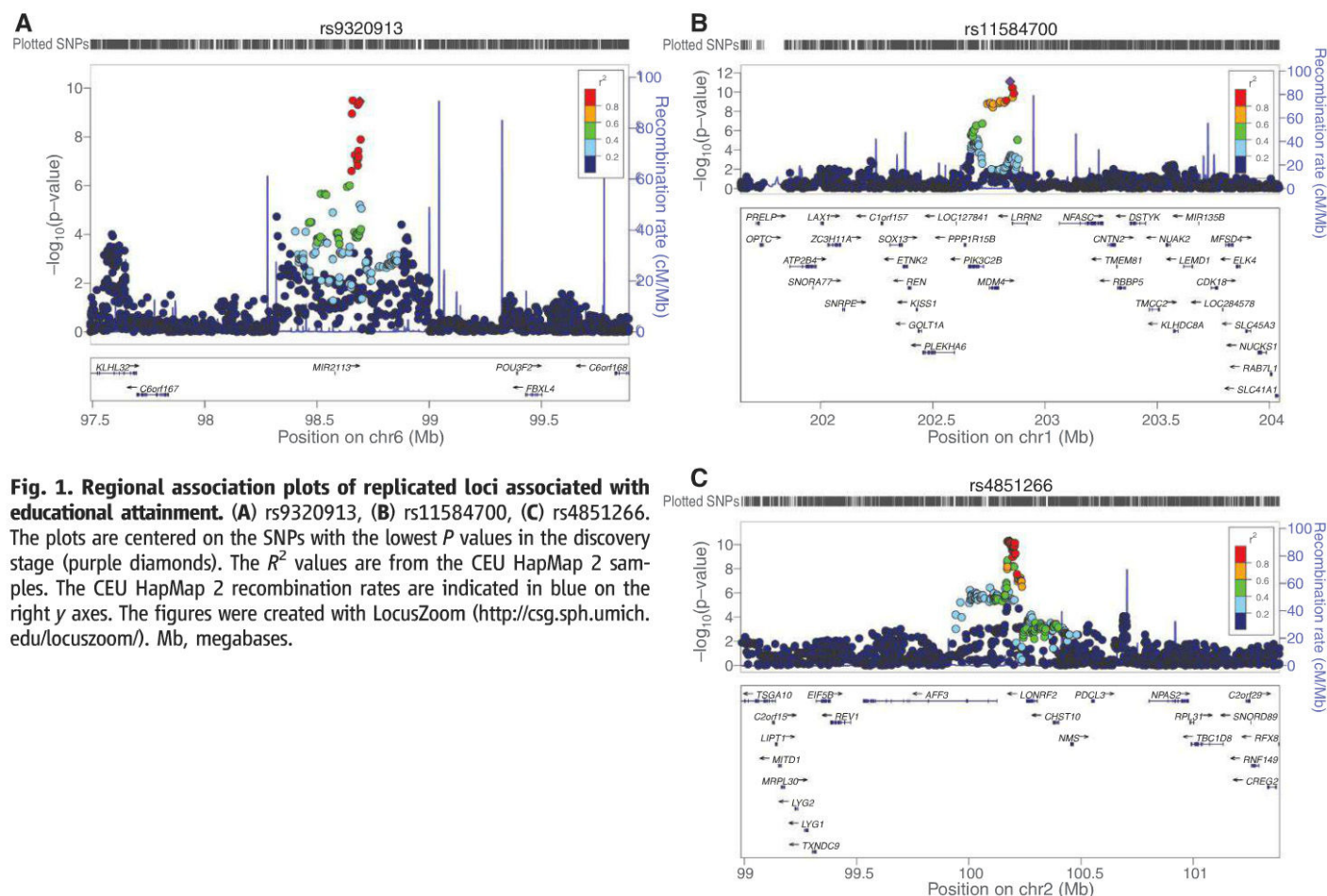
The polygenic score remains associated with educational attainment and cognitive function in within-family analyses (table S25). Thus, these results appear robust to possible population stratification.

If the size of the training sample used to estimate the linear polygenic score increased, the explanatory power of the score in the prediction sample would be larger, because the coefficients used for constructing the score would be estimated with less error. In (5), we report projections of this increase. We also assess, at various levels of explanatory power, the benefits from using the score as a control variable in a randomized educational intervention (5). An asymptotic upper bound for the explanatory power of a linear polygenic score is the additive ge-

netic variance across individuals captured by current SNP microarrays. Using combined data from STR and QIMR, we estimate that this upper bound is 22.4% (SE = 4.2%) in these samples (5) (table S12).

Placed in the context of the GWAS literature (10), our largest estimated SNP effect size of 0.02% is more than an order of magnitude smaller than those observed for height and body mass index (BMI): 0.4% (15) and 0.3% (18), respectively. For comparison with the R^2 value of 2% from our linear polygenic score for education, estimated from a sample of 120,000, a score for height reached 10%, estimated from a sample of 180,000 (15), and a score for BMI, using only the top 32 SNPs, reached 1.4% (18). Taken together, our findings suggest that the genetic architecture of complex behavioral traits is far more diffuse than that of complex physical traits.

Existing claims of “candidate gene” associations with complex social-science traits have reported widely varying effect sizes, many with R^2 values more than 100 times larger than those we have found (4, 6). For complex social-science phenotypes that are likely to have a genetic architecture similar to educational attainment, our estimate of 0.02% can serve as a benchmark for conducting power analyses and evaluating the plausibility of existing findings in the literature.



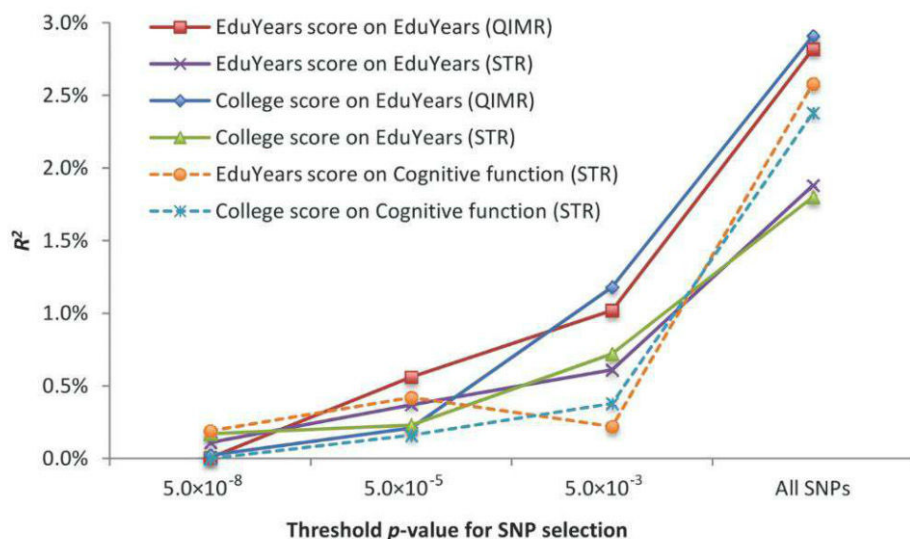


Fig. 2. Explanatory power of the linear polygenic scores estimated for EduYears or College. Solid lines show results from regressions of EduYears on linear polygenic scores in a set of unrelated individuals from the QIMR ($n = 3526$) and STR ($n = 6770$) cohorts. Dashed lines show results from regressions of cognitive function on linear polygenic scores in a sample from STR ($n = 1419$). The scores are constructed from the meta-analysis for either EduYears or College, excluding the cohort (either QIMR or STR) subsequently used as the prediction sample.

The few GWAS studies conducted to date in social-science genetics have not found genome-wide significant SNPs that replicate consistently (19, 20). One commonly proposed solution is to gather better measures of the phenotypes in more environmentally homogenous samples. Our findings demonstrate the feasibility of a complementary approach: identify a phenotype that, although more distal from genetic influences, is available in a much larger sample [see (5) for a simple theoretical framework and power analysis]. The genetic variants uncovered by this “proxy-phenotype” methodology can then serve as a set of empirically-based candidate genes in follow-up work, such as tests for associations with well-measured endophenotypes (e.g., personality, cognitive function), research on gene-environment interactions, or explorations of biological pathways.

In social-science genetics, researchers must be especially vigilant to avoid misinterpretations. One of the many concerns is that a genetic association will be mischaracterized as “the gene for X,” encouraging misperceptions that genetically influenced phenotypes are immune to environmental intervention [for rebuttals, see (21, 22)] and misperceptions that individual SNPs have large effects (which our evidence contradicts). If properly interpreted, identifying SNPs and constructing polygenic scores are steps toward usefully incorporating genetic data into social-science research.

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Supplementary Materials

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BigBrain: An Ultrahigh-Resolution 3D Human Brain Model

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Reference brains are indispensable tools in human brain mapping, enabling integration of multimodal data into an anatomically realistic standard space. Available reference brains, however, are restricted to the macroscopic scale and do not provide information on the functionally important microscopic dimension. We created an ultrahigh-resolution three-dimensional (3D) model of a human brain at nearly cellular resolution of 20 micrometers, based on the reconstruction of 7404 histological sections. “BigBrain” is a free, publicly available tool that provides considerable neuroanatomical insight into the human brain, thereby allowing the extraction of microscopic data for modeling and simulation. BigBrain enables testing of hypotheses on optimal path lengths between interconnected cortical regions or on spatial organization of genetic patterning, redefining the traditional neuroanatomy maps such as those of Brodmann and von Economo.

Brain organization on multiple scales and regional segregation are key elements for the development of a realistic model of the human brain. Multiscale organization requires the integration of both multilevel and multimodal data, at the level of cells with their specific connectivity to the level of cognitive systems and the whole brain. Magnetic resonance imaging (MRI) enables the study of the structure and function of the living human brain, with a spatial resolution in the range of 1 mm for structural imaging and somewhat larger for functional MRI (1, 2). This resolution is well above the cellular scale but has been sufficient for establishing human brain atlases to capture information at the level of brain areas, subcortical nuclei, gyri, and sulci (2–5). Cytoarchitectonic probabilistic maps enable the identification of microstructural correlates involved in a specific brain function, as determined by functional MRI, during a cognitive task, for example (6, 7). This approach is supported by combined physiological and imaging studies showing that the response properties of neurons change at the border of two areas (8, 9). Existing human brain atlases do not allow for the integration of information at the level of cortical layers, columns, microcircuits, or cells (fig. S1), such as has been shown recently for mouse or invertebrate brains (10, 11). Still, fine-grain anatomical resolution is a necessary prerequisite to fully understand the

neurobiological basis of cognition, language, emotions, and other processes, as well as to bridge the gap between large-scale neural networks and local circuitry within the cerebral cortex and subcortical nuclei.

We sought to create a human brain model at nearly cellular resolution by going considerably beyond the 1-mm resolution of presently available atlases, taking advantage of recent progress in computing capacities, image analysis, and relying on our experience in processing his-

tological sections of the complete brain. Major challenges include, but are not limited to, the highly folded cerebral cortex, the large number of areas, considerable variability among brains, and the sheer size of the brain, with its nearly 86 billion neurons and the same number of glial cells (12, 13). Compared with rodent or invertebrate brains, the human brain is extremely complex: For example, the volume of a human cerebral cortex is ~7500 times larger than a mouse cortex, and the amount of white matter is 53,000 times larger in humans than in mice. The recently published data set of the digitized mouse brain with 1- μ m resolution has a total amount of uncompressed volume data of 8 Tbyte (10). The creation of a volume with similar spatial resolution for the human brain would result in ~21,000 Tbyte. The interactive exploration (as opposed to simple storage) of such a data set is beyond the capacities of current computing. Thus, among other methodological problems, data processing becomes a major challenge for any project aiming at the reconstruction of a human brain at cellular resolution.

To create the brain model, we used a large-scale microtome to cut a complete paraffin-embedded brain (65-year-old female) coronally (Fig. 1), and we then acquired 7400 sections at 20- μ m thickness and stained them for cell bodies (14). Histological sections were digitized, resulting in images of maximally 13,000 by 11,000 pixels (10-by-10- μ m pixel size). The total volume of this

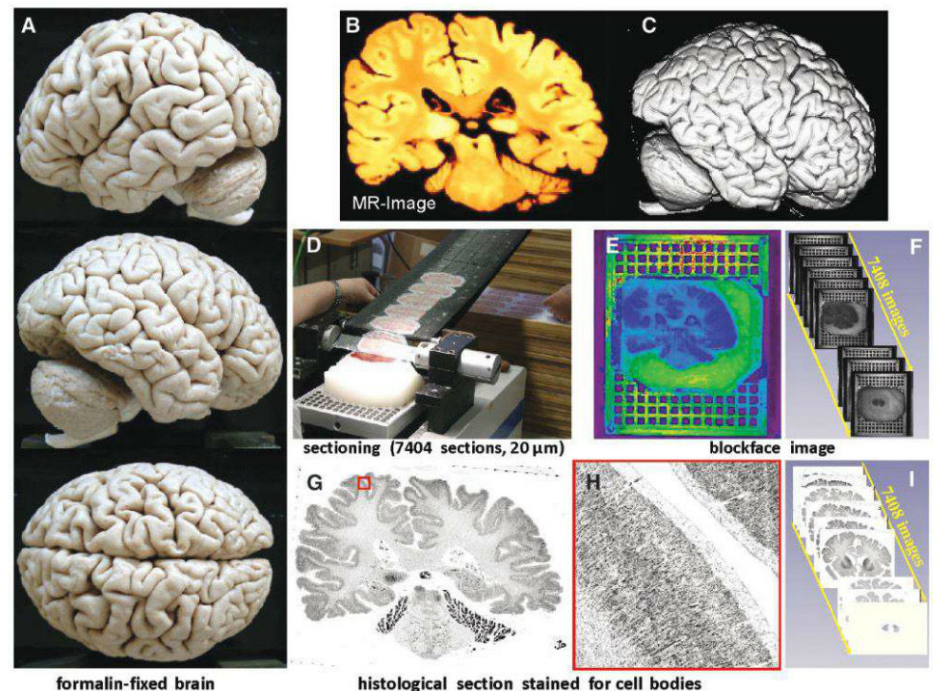


Fig. 1. Illustration of tissue and image processing. (A) Photographs of the fixed brain. Lateral left (top), lateral right (middle), and dorsal (bottom) views. (B) Magnetic resonance image (coronal view) and (C) 3D-reconstructed MRI volume of the fixed brain. (D) Histological sectioning. (E) Blockface image of a section (pseudocolored) resting on the mounting grid that served for alignment of the blockface images. (F) Series of blockface images. (G) Cell body-stained histological sections with the region of interest denoted by a red box. This area is shown with higher magnification in (H). (I) Series of histological images, which were 3D-reconstructed using the blockface images (F) and the MRI (C).

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data set was 1 Tbyte. The uninterrupted data acquisition time was ~1000 hours. To generate a data set with isotropic resolution, we down-scaled all images to 20 μm by 20 μm to match the section thickness of 20 μm .

Histological processing inevitably introduces artifacts, which pose problems at all stages of the three-dimensional (3D) reconstruction process. Defects include rips, tears, folds, missing and displaced pieces, distortion (shear), stain inhomogeneity, and crystallization. We performed both manual and automatic repairs to restore the integrity of all sections before the 3D reconstruction of the whole brain as a contiguous volume (figs. S2 to S4). The repaired sections were registered to the MRI, which served as an undistorted frame of reference, and further aligned section-to-section with the use of nonlinear registration. All calculations were carried out on high-performance computing (HPC) facilities within the Compute Canada network and were run on Jülich Research on Petaflop Architecture (JUROPA) at the Jülich Supercomputing Centre (see the supplementary materials).

Figure 2 shows three example regions from primary sensory and motor cortices in the original coronal plane and the reconstructed sagittal and horizontal planes. Note the smooth contours

in the virtual sections, confirming the high quality of the 3D reconstruction. The images in all three planes at 20 μm reveal differences in the laminar pattern between brain areas and enable an observer-independent definition of borders between them (15). To prove the feasibility of our mapping approach in higher associative cortices with more subtle architectonic differences in between, we defined a border between Brodmann area (BA) 10 of the frontal pole and BA32 (Fig. 3). Although some artifacts due to residual mismatches between aligned sections still exist, the border of interest has been detected in the original and the horizontal virtual plane at the identical location. Thus, the present “BigBrain” model allows the recognition of not only the borders between primary cortical areas [feasible, at least to some extent with advanced MRI technology (16–19)], but also between higher associative areas. Until now, the recognition of the latter borders, based on their laminar pattern, was accessible in 2D histological sections and light-microscope images, but at only those locations where the cortex was cut orthogonal to the pial surface. The latter condition is often not fulfilled (e.g., Fig. 2A, coronal), thus making border definition based on quantitative criteria throughout the whole cortical ribbon in 2D sections impossible.

The 3D-analysis indicates that the relationship between cortical folds and borders of cytoarchitectonic areas is heterogeneous. Whereas this relationship is considerably close for some areas, it seems to be less well defined for others. For example, the border between the primary motor and somatosensory cortex is localized in the fundus of the central sulcus, independently from the orientation of the cutting plane (Fig. 2A). This is not the case for the primary auditory area Te1 (BA41), which is more or less restricted by Heschl’s gyrus in two planes (Fig. 2C), but has no sulcal landmark in the third plane (20). Whereas the sulcal pattern is associated with areal borders in other, nonprimary areas [for example, BA35 (21)], the border between the primary visual area BA17 (V1) and neighboring 18 (V2) (Fig. 2B) does not seem to be related to a sulcus. The same is true for the border between BA10 and the neighboring cingulate cortex (Fig. 3). This variable relationship between cytoarchitectonic borders and macroscopic landmarks has been analyzed in the past (22, 23), but not in 3D space.

The spatial dimension, however, is relevant, because the directionality of hemispheric growth during embryonic and fetal development and the coupling of cortical areas via fiber tracts define the spatial organization of cortical areas and their connections, as well as sulci and gyri in the adult brain. The effect of early cortical regionalization on folding has been modeled by introducing geometric, mechanic, and growth asymmetries in the model (24). Another model considered variability between brains during ontogeny (25). A recent study has emphasized the strong geometric structure of fibers and pathways as a result of early development (26). Another study reported that fiber connection patterns closely follow gyral folding patterns in the direction tangential to the cortical sphere (27). The concept of the tension-based morphogenesis effect provides a theory of folding processes caused by the tension of fiber tracts connecting brain regions (28, 29), whereas others identify differences in the relationship between supra- and infragranular layers (i.e., cytoarchitectonic differences) as factors shaping cortical folding (30). The validation of all these concepts requires high-resolution spatial models of the human brain for testing the underlying hypotheses.

The present findings and data on the localization of cortical areas with respect to gyri and sulci support the notion that their topographical relationship is not merely a pure geometric phenomenon, but rather the result of an interference of developmental processes and the internal structure of areas, including their connectivity (31). A systematic analysis of cortical borders across the whole cortical ribbon is mandatory. The variability in this relationship across individuals requires the generation of additional BigBrain data sets in the future, labor-intensive work that is currently underway.

To consider intersubject variability in the present data set, vector fields have been calculated

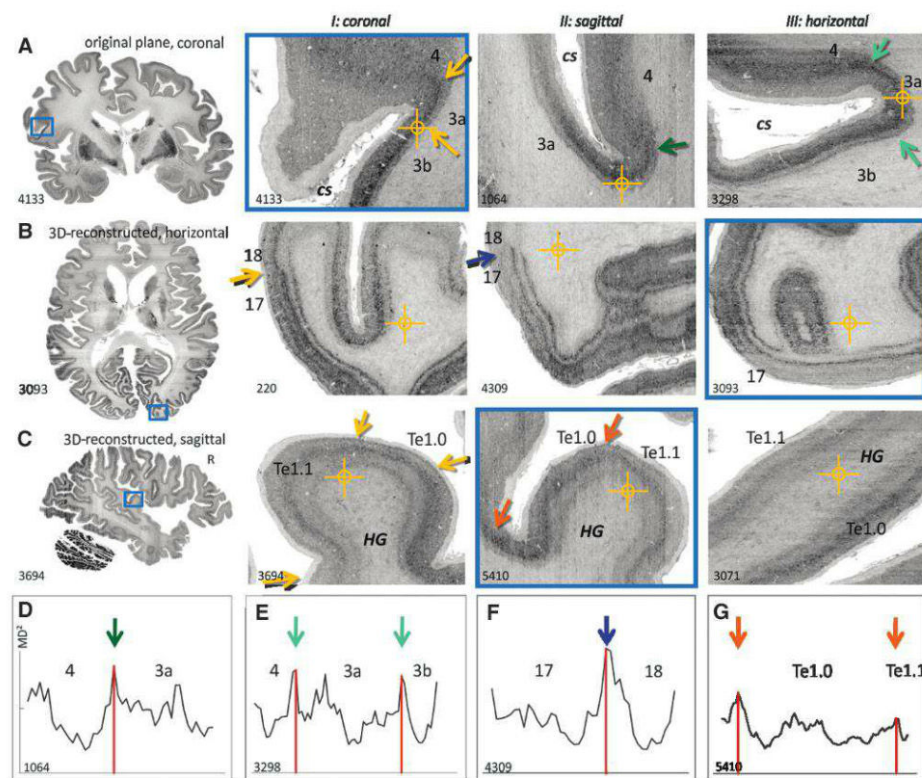


Fig. 2. Primary cortical regions in the three planes of section. (A) Sensorimotor (BA4, 3a and 3b), (B) visual [BA17 (V1) and BA18 (V2)], and (C) auditory cortex (areas Te1.0, Te1.1 as part of BA41) (left column). Overviews of the whole-brain sections in the original plane (A) and the 3D-reconstructed horizontal (B) and sagittal (C) planes. Crosshairs denote identical positions within a row. Columns I to III show coronal, sagittal, and horizontal planes, respectively. cs, central sulcus; HG, Heschl’s gyrus. Section numbers are shown in the lower-left corner of each panel. (D to G) Definition of borders for regions of interest from (A) to (C), based on the Mahalanobis distance (15). Corresponding borders are labeled by identically colored arrows (see also supplementary materials and Fig. 3).

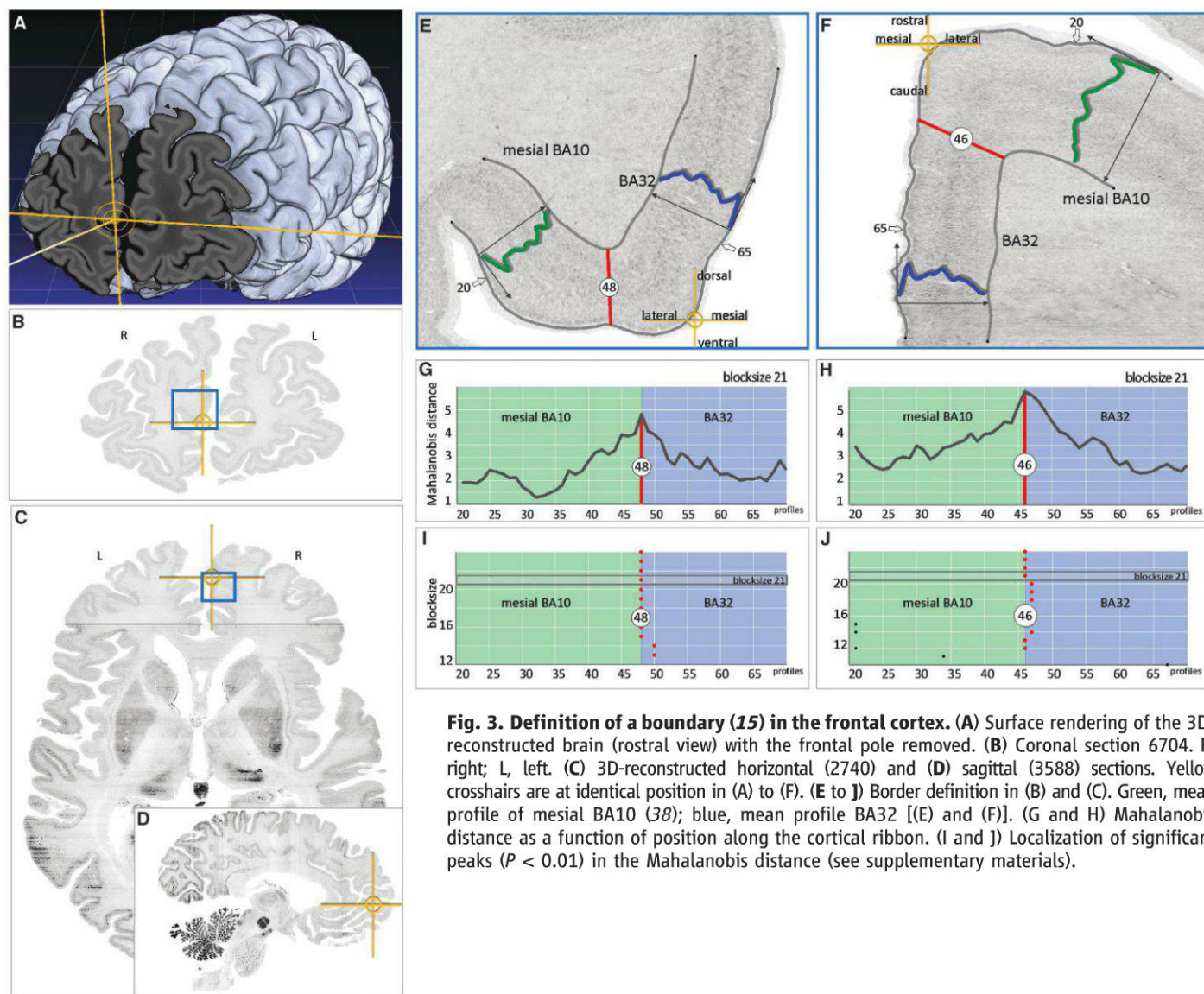


Fig. 3. Definition of a boundary (15) in the frontal cortex. (A) Surface rendering of the 3D-reconstructed brain (rostral view) with the frontal pole removed. (B) Coronal section 6704. R, right; L, left. (C) 3D-reconstructed horizontal (2740) and (D) sagittal (3588) sections. Yellow crosshairs are at identical position in (A) to (F). (E to J) Border definition in (B) and (C). Green, mean profile of mesial BA10 (38); blue, mean profile BA32 [(E) and (F)]. (G and H) Mahalanobis distance as a function of position along the cortical ribbon. (I and J) Localization of significant peaks ($P < 0.01$) in the Mahalanobis distance (see supplementary materials).

based on a 400- μm isotropic down-sampled volume, to define a homeomorphic transformation between the BigBrain and the Montreal Neurological Institute (MNI) space (see supplementary materials). Thus, cytoarchitectonic or functional probability maps in MNI space, which embeds information about intersubject variability, can be mapped to the BigBrain data set. We plan to establish links to other reference systems so as to combine high-resolution cytoarchitectonic data with, for example, gene-expression maps (32), neural projections (33) or future brain-activity maps (34).

The BigBrain data set will be made publicly available to promote the development of new tools for defining 3D cytoarchitectonic borders (<http://bigbrain.cbrain.mcgill.ca>). BigBrain allows the extraction of parameters of cortical organization by enabling measurements parallel to cell columns (e.g., cortical thickness, densities of cell bodies per column, surface measures) to pro-

vide a “gold standard” for calibrating in vivo measurements of cortical thickness and other measures.

The BigBrain data set represents a new reference brain, moving from a macroanatomical perspective to microstructural resolution. This model provides a basis for addressing stereotaxic and topological positions in the brain at micrometer range (e.g., with respect to cortical layers and sublayers). BigBrain will make it possible to localize findings obtained in cellular neuroscience and mapping studies targeting transmitter receptor distributions (35), fiber bundles (36), and genetic data (32, 37). The BigBrain model can also be exploited as a source for generating realistic input parameters for modeling and simulation. It thus represents a reference frame with nearly cellular resolution, a capability that has not been previously available for the human brain, while considering the regional heterogeneity of human brain organization.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/340/6139/1472/DC1
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Compartmentalized Calcium Transients Trigger Dendrite Pruning in *Drosophila* Sensory Neurons

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Dendrite pruning is critical for sculpting the final connectivity of neural circuits as it removes inappropriate projections, yet how neurons can selectively eliminate unnecessary dendritic branches remains elusive. Here, we show that calcium transients that are compartmentalized in specific dendritic branches act as temporal and spatial cues to trigger pruning in *Drosophila* sensory neurons. Calcium transients occurred in local dendrites at ~3 hours before branch elimination. In dendritic branches, intrinsic excitability increased locally to activate calcium influx via the voltage-gated calcium channels (VGCCs), and blockade of the VGCC activities impaired pruning. Further genetic analyses suggest that the calcium-activated protease calpain functions downstream of the calcium transients. Our findings reveal the importance of the compartmentalized subdendritic calcium signaling in spatiotemporally selective elimination of dendritic branches.

Nervous system development relies on a balance between progressive and regressive events (1, 2). After progressive events such as axon and/or dendrite outgrowth and synapse formation, neurons refine their connections through regressive events, such as pruning of axons and dendrites (1–3). In dendrite pruning, neurons selectively eliminate excessive or inappropriate connections that formed during development. Thus, proper dendrite pruning depends on local activation of the machinery needed to eliminate unnecessary dendritic branches; however, our understanding of locally acting mechanisms involved in this process remains incomplete.

Drosophila dendrite arborization sensory neurons are classified into four subtypes (I to IV) on

the basis of their dendritic morphology, and class IV dendrite arborization (C4da) sensory neurons undergo dendrite pruning during early metamorphosis (3–7). C4da dendrites are typically severed at the proximal regions ~5 hours after puparium formation (APF), followed by fragmentation and clearance of the severed branches by 14 hours APF (4, 5). Previous studies have demonstrated that the ubiquitin-proteasome system and the caspase activity act to execute the pruning process (5–7), but it remains unclear what mechanisms locally determine which dendritic branches are to be eliminated.

Calcium signaling is often utilized for the spatiotemporal regulation of neural development (2, 3). To monitor changes in intracellular Ca^{2+} ion concentration ($[\text{Ca}^{2+}]_i$), we expressed the genetically encoded calcium (Ca^{2+}) indicator GCaMP3 selectively in C4da neurons using the Gal4/UAS binary system (which exploits the yeast Gal4 transcriptional activator and the upstream activating sequence) (fig. S1) (8). We continuously monitored $[\text{Ca}^{2+}]_i$ levels in single C4da neurons from 1 to 8 hours APF and found no apparent $[\text{Ca}^{2+}]_i$

changes in soma and axons (9). However, we observed robust Ca^{2+} transients in groups of dendrites, which typically started 2 to 4 hours APF (Fig. 1, A to C, and movie S1). The dendritic Ca^{2+} transients appeared with a frequency of $0.88 \pm 0.66 \text{ min}^{-1}$ on average ($n = 36$), and the mean change in fluorescence ($\Delta F/F_0$) of these events was $499.1 \pm 162.5\%$ (99 events, $n = 10$). Once Ca^{2+} transients commenced in a particular group of dendrites, transients continued to occur in the same dendrites. Furthermore, patterns, as well as amplitudes, of Ca^{2+} transients observed in individual dendritic groups were not correlated to each other (Fig. 1D and movie S1), which suggested that the Ca^{2+} transients are compartmentalized into each subdendritic region. Hereafter, we designate the groups of dendritic branches with the compartmentalized Ca^{2+} transients as "branch units." Dendritic arbors in single neurons were typically subdivided into 4 to 5 branch units, which were mainly formed in dendrites distal from the secondary and from the tertiary branches (fig. S2).

An example of long-term simultaneous monitoring of $[\text{Ca}^{2+}]_i$ and dendritic arbor structures by GCaMP3 and membrane-bound red fluorescent protein (RFP), respectively, is shown (Fig. 2). The first branch unit started its Ca^{2+} transients 2 hours APF, when no obvious pruning was detected (Fig. 2, C and D, and movie S2). The second and third branch units started their Ca^{2+} transients around 3.5 and 4.5 hours APF, respectively, when all the dendritic branches seemed still intact (Fig. 2E and movies S3 and S4). Five hours APF, the first branch unit underwent fragmentation after a continuous Ca^{2+} influx, whereas the other two still continued their Ca^{2+} transients, and their dendritic arbors seemed intact (Fig. 2F). Similarly, the second and third branch units were eliminated after continuous Ca^{2+} influx about 6.5 and 7.5 hours APF, respectively (Fig. 2, G and H). Thus, dendrites are pruned in the temporal order in which they experienced Ca^{2+} transients, the mean duration of which was $140.5 \pm 43.6 \text{ min}$ ($n = 11$). The location and timing of the occurrence of Ca^{2+} transients correlated perfectly with those of dendrite pruning ($n \geq 50$ neurons),

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which indicated that the Ca^{2+} transients in branch units spatially and temporally predict the dendritic branches to be pruned within several hours.

Neuronal Ca^{2+} signaling involves an intricate interplay between Ca^{2+} influx across the plasma membrane and Ca^{2+} release from the endoplasmic reticulum (ER) stores (10). We next conducted a candidate screen to identify the Ca^{2+} influx pathways responsible for generating the Ca^{2+} transients (tables S1 and S2). Both knockdown and knockout of *ca-beta*, the auxiliary β subunit of the voltage-gated Ca^{2+} channels ($\text{Cav}\beta$) (11), resulted in a significant reduction in the amplitude and frequency of Ca^{2+} transients (Fig. 3, A and B; table S1; and fig. S3). These defects in *ca-beta* knockout clones [generated by the mosaic analysis with a repressible cell marker (MARCM) technique (12)] were fully rescued by restoring *ca-beta* expression in C4da neurons (Fig. 3B), which confirmed the cell-autonomous function of *ca-beta*. Furthermore, Ca^{2+} transients were completely abolished in neurons lacking functional copies of both *ca- α 1D* and *cacophony* (*cac*), the *Drosophila* homologs of the α 1 subunit $\text{Cav}1$ (L-type) and $\text{Cav}2$ genes (P/Q-, N-, and R-type), respectively, whereas the frequencies were only partially reduced in the single-mutant clones of *ca- α 1D* or *cac* (Fig. 3, A and B). Thus, the voltage-gated Ca^{2+} channels (VGCCs) are responsible for generating the compartmentalized Ca^{2+} transients.

What mechanisms can induce Ca^{2+} transients in such a compartmentalized manner? One possibility is that intrinsic dendritic excitability increases locally in individual branch units, which thereby sensitizes the VGCCs-mediated Ca^{2+} influx. To test this, we designed an experiment to optogenetically assess and manipulate dendritic excitability (Fig. 3C; see Methods in the supplementary materials for details). Briefly, we expressed the channelrhodopsin-2 (ChR2), a cation channel gated by blue light (13) and the Ca^{2+} indicator RGECO1 (14) in C4da neurons, and measured the threshold of the blue light intensity required to elicit dendritic Ca^{2+} influxes, which we confirmed to be dependent on the VGCCs opening in response to ChR2 activation (fig. S4). One hour APF, the threshold intensity was defined to be 1.1 mW mm^{-2} (median, $n = 13$ neurons) (Fig. 3D, left). Typically, 2 to 3 hours APF, branch excitability was elevated in local dendrites, as the threshold intensities were reduced by a factor of three in a group of dendrites (a median of 0.44 mW mm^{-2}) (Fig. 3D, right) but not in other branches (fig. S4). In all cases, the dendritic branches with elevated excitability commenced “spontaneous” Ca^{2+} transients and were eventually eliminated within 3 hours (fig. S4), which suggested that the dendritic branches with elevated excitability correspond to branch units. Thus, our data indicate that intrinsic excitability of dendritic branches is locally elevated at the early pupal stage, which presumably promotes the robust VGCC-mediated Ca^{2+} influx in branch units. The increment of the branch excitability

is regulated in part by the ecdysone signaling in C4da neurons, as cell-autonomous suppression of the ecdysone signaling blocked the occurrence of Ca^{2+} transients (fig. S5).

To test whether Ca^{2+} transients are required for dendrite pruning, we examined the pruning phenotypes in VGCCs mutant neurons. In MARCM clones, homozygous for *ca-beta* and doubly homozygous for *cac* and *ca- α 1D*, dendrite pruning was significantly compromised (Fig. 4A), although there were no obvious defects in development of larval dendrites (fig. S6). No apparent defects in microtubule destabilization or dendritic severing at proximal regions were observed in *ca-beta* mutant neurons (fig. S7), which suggested that Ca^{2+} transients seem to be dispensable for these events. The pruning defects in *ca-beta* mutants were largely rescued

by restoring the expression of *ca-beta* in C4da neurons (Fig. 4A). To inhibit the VGCC-mediated Ca^{2+} influx specifically during metamorphosis, we temporally expressed a hyperpolarizing Kir2.1 potassium channel (15) in C4da neurons by using the flip-out technique, and we found that in the flip-out clones generated by a heat shock treatment at 24 hours before the onset of metamorphosis, dendrite pruning was significantly inhibited (fig. S8). Thus, the VGCC-mediated Ca^{2+} influx is required in metamorphosis for dendrite pruning.

Local caspase activation in dendrites is reported to be important for removal of C4da dendrites (6, 7). Given that caspase activation was predominantly detected after 7 hours APF, which is after Ca^{2+} transients were observed in most C4da neurons (fig. S9), caspases might function downstream of the Ca^{2+} signaling. However,

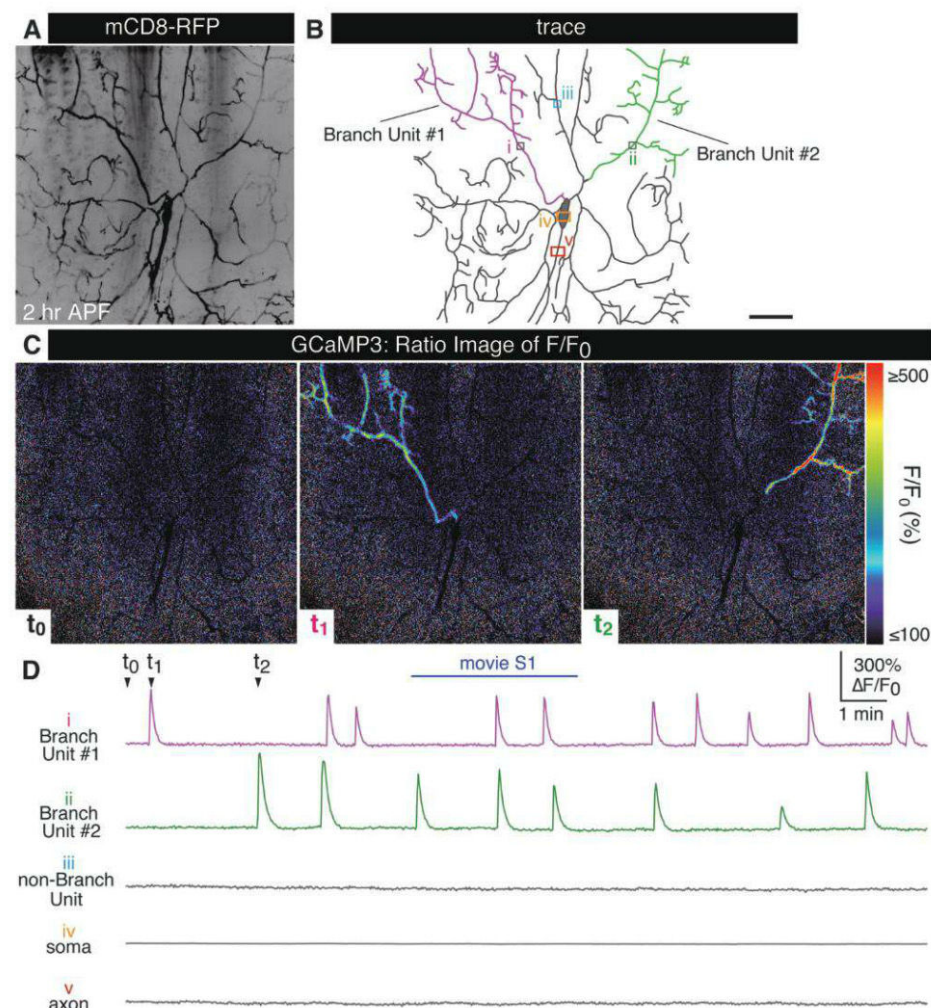


Fig. 1. Compartmentalized Ca^{2+} transients occur in C4da dendrites during early metamorphosis. (A and B) Live confocal image (A) and corresponding schematic trace (B) of a C4da neuron 2 hours APF labeled with mCD8 fused with red fluorescent protein (mCD8-RFP). Dendritic branches corresponding to branch units 1 and 2 are shown in magenta and green, respectively. On the schematic trace, i, ii, iii, iv, and v correspond to the places where we measured the calcium dynamics [see legend of (D)]. (C) Ratiometric images of GCaMP3 fluorescence at three time points (t_0 , t_1 , and t_2). Each time point is indicated with arrowheads in (D). (D) Time traces of GCaMP3 fluorescence in five different regions, the locations of which are indicated in (B) (fractional change in fluorescence, $\Delta F/F_0$). A time-lapse movie corresponding to the blue time bar is available as movie S1. Scale bar, $50 \mu\text{m}$.

comparable levels of caspase activation were detected in *ca-beta* mutant dendrites (fig. S10). In addition, we also found no obvious defects in the occurrence of the dendritic Ca^{2+} transients in neurons mutant for the initiator caspase *dronc* (fig. S11). It is thus likely that the caspase is not the major effector of the Ca^{2+} signaling and that the Ca^{2+} transients and the caspase activity function cooperatively to promote dendrite pruning. Consistently, pruning defects were synergistically enhanced in *ca-beta; dronc* double-mutant neurons compared with those in *ca-beta* or *dronc* single-mutant neurons (fig. S12).

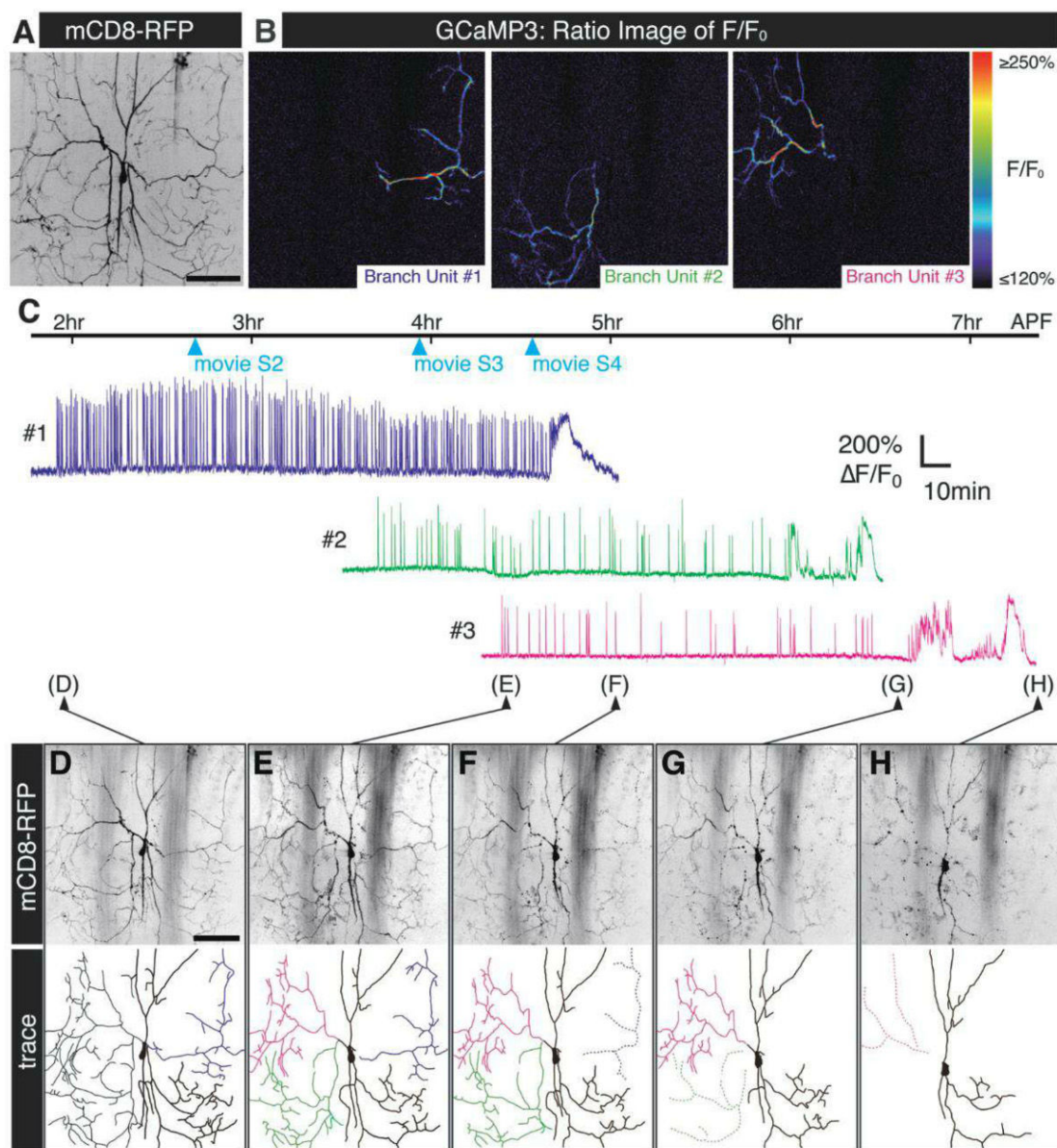
To gain insight into the molecular mechanism underlying how Ca^{2+} transients promote dendrite pruning, we examined the pruning phenotypes after knockout or knockdown of individual genes whose functions are controlled by the intracellular Ca^{2+} (table S3). Among the 17 genes tested, dendrite pruning was partially, but significantly,

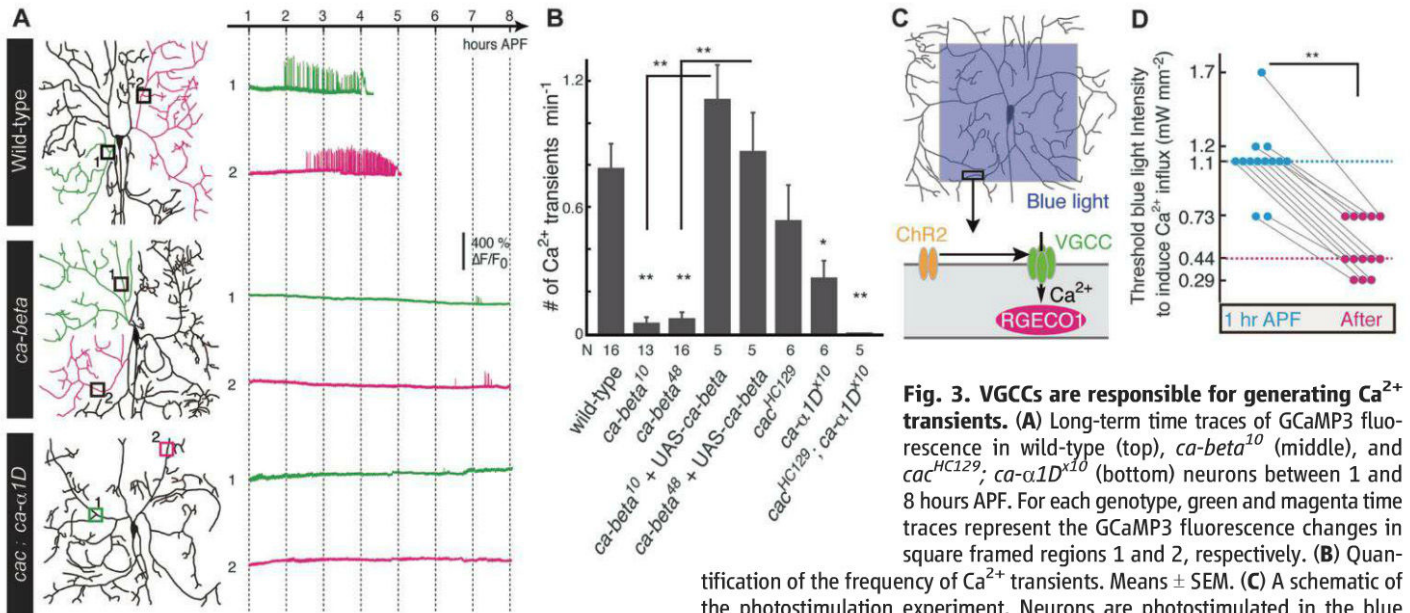
attenuated in the Ca^{2+} -activated protease calpain *calpA* mutants, and this attenuation was largely rescued by restoring the expression of *calpA* in C4da neurons (Fig. 4B). The pruning defects in *calpA* mutants were significantly enhanced by a mutation in *calpB*, the other member of the two fly homologs of the conventional calpain, which resulted in pruning defects comparable to those in VGCC mutants (Fig. 4B). We tested for genetic interactions between calpains and VGCCs and found that pupae heterozygous for both *ca-beta* and two calpain genes showed significant pruning defects, whereas each one of them separately was normal (Fig. 4B), which suggested that the calpains function downstream of Ca^{2+} transients to promote dendrite pruning.

We have shown that Ca^{2+} transients occur locally in dendrites to trigger dendrite pruning in *Drosophila* sensory neurons. These compartmentalized Ca^{2+} transients are, to our knowl-

edge, the earliest events that occur in the dendritic branches that are destined to be pruned. Although many of previous studies have focused on pathological roles of the calpains (16), our findings suggest that, during developmental dendrite pruning, the calpain activities are developmentally regulated and are likely compartmentalized in unnecessary dendrites. We speculate that such controlled and localized calpain activations are accomplished by an intrinsic property of neurons—that is, by local increase in branch excitability to induce local Ca^{2+} transients. Compartmentalized changes in dendritic branch excitability are also observed in many different types of mammalian neurons (17, 18). It will be intriguing to examine whether compartmentalized Ca^{2+} transients and subsequent calpain activations are part of a conserved mechanism to trigger dendrite pruning. Future studies deciphering the role of dendritic Ca^{2+} transients will provide insights into the regulation of dendrite pruning.

Fig. 2. The occurrence of Ca^{2+} transients spatially and temporally correlates with dendrite pruning. (A) Live confocal image of a C4da neuron 0 hour APF. (B) Three branch units are visualized with ratiometric images of GCaMP3 fluorescence. (C) Long-term time traces of GCaMP3 fluorescence in branch unit 1 (blue), 2 (green), and 3 (magenta). Time-lapse movies S2 to S4 correspond to the light blue arrowheads. (D to H) Live confocal images and corresponding schematic traces at five different time points [indicated with arrowheads in (C)]. Schematic traces of each branch unit are shown in the same colors as in (C). Scale bars, 100 μm .





dendritic Ca^{2+} responses. Data points from the same neurons are connected with lines. Dotted lines, median values. **P* < 0.05; ***P* < 0.001; analysis of variance (ANOVA), Dunnett's post hoc test (B); paired *t* test (D).

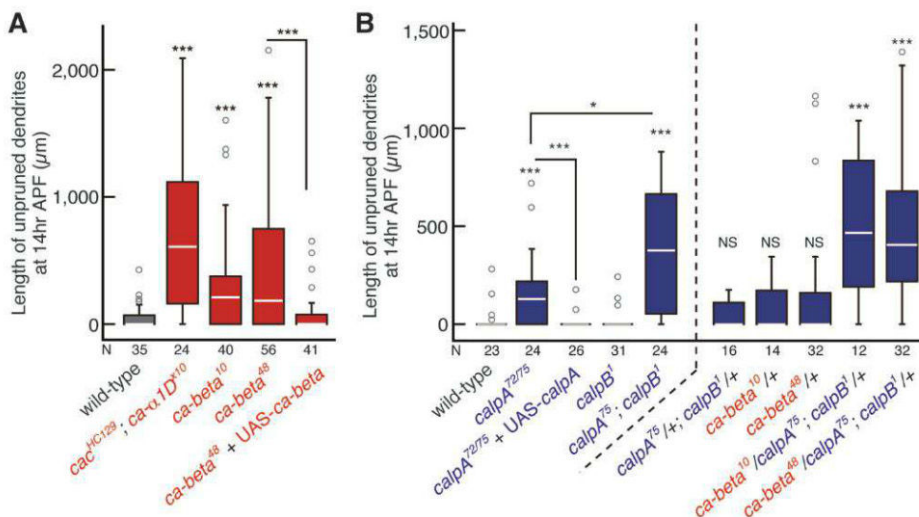


Fig. 4. Ca^{2+} transients are required for dendrite pruning. (A) Quantification of the pruning defects in the VGCCs mutant MARCM clones. Box plots indicate the median (white line), 25th, and 75th percentiles (box); the data range (whiskers); and outliers (circles). Outliers are defined as data points greater than the 75th percentile of all data points plus 1.5 times the interquartile range. (B) Quantification of the pruning defects in the calpain mutants. **P* < 0.05; ****P* < 0.001. NS, not significant.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1234879/DC1
Materials and Methods
Figs. S1 to S12
Tables S1 to S3
References (19–39)
Movies S1 to S4

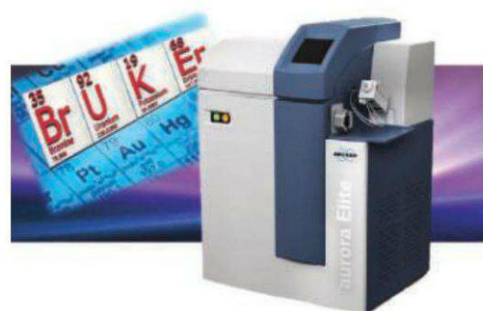
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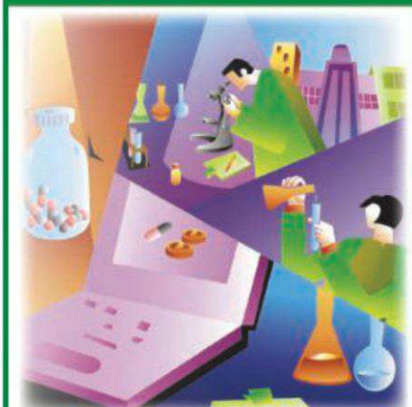
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The successful candidate will be actively involved in basic research on regulatory mechanisms of cellular biology in the area of Molecular Genetics. Applicants should complement and extend the existing strengths of the ZMB (<http://www.uni-due.de/zmb>) and the Faculty of Biology (<http://www.uni-due.de/biologie>).

Candidates are expected to establish an independent extramurally funded, competitive research program and to participate in teaching within the programs of the Faculty of Biology.

According to §36 of the NRW Act (Institutions of Higher Learning) the requirements for this position are a university degree, doctorate, additional scientific achievements attained in the context of a junior professorship, post-doctoral thesis, scientific work at a university, research institution, in business, administration or in another relevant area.

The University of Duisburg-Essen seeks to increase the number of female academic staff and encourages women with the necessary qualifications to apply for this position.

Given equal suitability for the appointment, the applications of disabled candidates will be given priority.

Applications, including the usual supporting documents (curriculum vitae, list of scientific publications, present and future research plans, information on teaching experience to date, involvement in academic self-administration and information about third-party funding) and the application form (available on <http://www.uni-due.de/zmb/application-w3-genetics>) should be submitted preferably by e-mail (**dekanat@biologie.uni-due.de**) within a period of 6 weeks after publication of this advertisement to the **Dekan der Fakultät für Biologie der Universität Duisburg-Essen, Univ.-Prof. Dr. Daniel Hoffmann, Universitätsstr. 2, 45141 Essen, Germany.**

Further information can be obtained from the head of the ZMB, Univ.-Prof. Dr. Michael Ehrmann, Tel. +49 201 183 2949, e-mail: michael.ehrmann@uni-due.de
www.uni-due.de



华中农业大学
HUAZHONG AGRICULTURAL UNIVERSITY

Recruitment for Dean of Life Science and Technology at Huazhong Agricultural University

Huazhong Agricultural University is one of the top agricultural universities, and a member of "the 211 Project" directly under the supervision of the Ministry of Education, China. Located in Wuhan, the campus covers an area of 495 hectares with well-spaced teaching blocks and laboratory buildings. It is surrounded on three sides by South Lake and backed by Lion Hill, which makes it an ideal place for teaching, researching and studying.

The College of Life Science and Technology encompasses two national key disciplines: Biochemistry & Molecular Biology and Microbiology, and includes two national talent training bases: Biology and Biotechnology. The National Center of Plant Gene Research (Wuhan), the National Key Laboratory of Genetic Crop Improvement and the National Key Laboratory of Agro-microbiology are also based in the College. The faculty of the College includes one academicien of the Chinese Academy of Sciences, five National "1000 Talent Plan" Professors, five Yangtze River Scholar Professors, four recipients of Young Investigator Award by the National Natural Science Foundation of China.

The College is seeking an outstanding scientist to take the position of the Dean starting January 1, 2014. The Dean is expected to take a leadership role toward the development of a college with an international reputation by building world-class education and research programs in life sciences. Applicants should be academics with (i) an outstanding record of scholarship in a discipline of life sciences; (ii) experience in leading well-funded and internationally-recognized research activities; (iii) demonstrated leadership and management ability at an relevant level in higher education institutions; (iv) long-term vision for future development of education and scientific research in the general area of life sciences; and (v) excellent interpersonal and communication skills. The selected candidate will be offered a full professorship in his/her own specialized area, together with the position of the Dean. The selected candidate is also anticipated to set up a research team which can continuously carry out cutting-edge research in a specific area of life sciences. The application of a team headed by a distinguished scientist is also encouraged.

The appointment is for five years with the option to extend by mutual agreement. Salary will be highly competitive which may be up to RMB 1 million per year, commensurate with qualifications and experience. The University offers a full package of fringe benefit including medical care, settling-in and housing allowance. The University will also provide research start-up fund, laboratory and research group support, with specifics negotiable during interview.

The following materials should be provided in the application package:

1. A full CV; copy of degree certificates; proof of employment; achievements, honors and awards which would represent the candidate;
2. List of five reference persons and their contact information;
3. Work plan (both with the college and in the major field of study)

Deadline: 2013/07/31(To the local postmark)

Search Procedure

1. August, 2013: Reviewing resumes of the applicants;
2. September 2013: Interviews
3. October 2013: Announcing the final result; signing the contract;
4. December 2013: Starting work.

For more recruitment information, please visit: www.hzau.edu.cn

Contact: Zhou Xiong

Tel: (+86)27-87286893

Fax: (+86)27-87384670

E-mail: zhouxiong@mail.hzau.edu.cn;

Mailing Address:

Personnel Office of Huazhong Agricultural University,
No.1 Shizishan Street, Hongshan District,
Wuhan, Hubei Province, 430070, P.R.China

Search Committee of Huazhong Agricultural University



Cancer Immunology and Inflammation Scientists

Georgia Regents University Cancer Center is undergoing an unprecedented expansion in its basic and population sciences programs as part of an initiative to achieve designation as an NCI Cancer Center. Following the successful completion of Phase 1 of this expansion, we now invite applications for positions at **Assistant, Associate and Full Professor** levels from interested individuals having expertise in **inflammation, immunology, metabolic control of immunity, tolerance and immunotherapy relating to the tumor microenvironment and tumor immunotherapy.**

Applicants must have active extramural research funding (preferably from NCI) and a strong track record of independent research. Ideal candidates will have conducted research in an NCI-designated Cancer Center. Successful applicants will join a collaborative Program that works closely with clinical research oncologists to promote translational research.

A competitive salary and start-up package are available, commensurate with experience and academic qualifications. A summary of research interests, curriculum vitae and names of three references should be sent to **Dr. Rhea-Beth Markowitz, rbmarkowitz@gru.edu.**

The GRU Cancer Center and the University System of Georgia are Equal Employment Opportunity/Affirmative Action Employers.



Basic Cancer Research Scientists

Georgia Regents University Cancer Center is undergoing an unprecedented expansion in its basic and population sciences programs as part of an initiative to achieve designation as an NCI Cancer Center. Following the successful completion of Phase 1 of this expansion, we now invite applications for positions at **Assistant, Associate, and Full Professor** levels from interested individuals having expertise in **obesity-related cancer research, tumor angiogenesis, HPV and cancer, genomics and personalized medicine.**

Applicants must have active extramural research funding (preferably from NCI) and a strong track record of independent research. Ideal candidates will have conducted research in an NCI-designated Cancer Center. Successful applicants will join a collaborative Program that works closely with clinical research oncologists to promote translational research.

A competitive salary and start-up package are available, commensurate with experience and academic qualifications. A summary of research interests, curriculum vitae, and names of three references should be sent to **Dr. Rhea-Beth Markowitz, rbmarkowitz@gru.edu.**

The GRU Cancer Center and the University System of Georgia are Equal Employment Opportunity/Affirmative Action Employers.



Opportunities for POSTDOCTORAL RESEARCHERS in Biomedical Research at the Spanish National Centre for Cardiovascular Research CNIC, Madrid - Spain

The CNIC is dedicated to excellence in cardiovascular research and to translating new knowledge into real improvements in clinical practice.

The scientific project of the centre has been structured in three areas:

- Cardiovascular Development and Repair Department (CDR)
- Vascular Biology and Inflammation Department (VBI)
- Epidemiology, Atherothrombosis and Imaging Department (EAI)

To be eligible, candidates must:

- Hold a PhD degree that must have been awarded no more than five years ago (exceptions will be made for documented career breaks and candidates with children)
- Have, at least, one publication as first author in an international peer reviewed journal
- Candidates must not have resided or carried out their main activity in Spain for more than twelve months in the last three years

The CNIC can offer you:

- A 3-year contract
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- Scientific-technical support and complementary training

Deadline for submission of proposals: 30 August 2013.

CNIC is an inclusive, equal opportunity employer, irrespective of nationality, ethnic origin, gender, marital or parental status, sexual orientation, creed, disability, age or political belief. Confidentiality is guaranteed throughout the selection process and all current regulations relating to the protection of personal data will be strictly adhered to.

For further information and applications, please, visit www.cnic.es



Faculty Position in Developmental/Cancer Biology

The Department of Anatomy and Regenerative Biology at The George Washington University School of Medicine and Health Sciences (SMHS) invites applications for a tenure-track or tenured position at the rank of Assistant or Associate Professor. We seek an outstanding candidate with demonstrated significant contributions in the areas of developmental signaling networks and stem cell self-renewal pathways; somatic cell reprogramming which has parallels with malignant transformation; or epithelial-to-mesenchymal transition and tumor-propagating cells.

Basic Qualifications: Applicants must have a Ph.D. and/or M.D. degree, a record of top-tier publications, current extramural funding and a history of collaborative research.

Current research in the department focuses on normal and malignant aspects of epithelial, hematopoietic and nervous systems; and DNA damage/stress responses and drug resistance in cancer and cancer therapy. The SMHS is centrally located on GW's Foggy Bottom campus; ample opportunities exist to collaborate with cancer researchers in other SMHS departments, in basic and applied science departments on campus, at the NIH, Children's National Medical Center, and at other universities in the Washington DC metropolitan area; and to participate in the establishment of a new cancer research center.

Competitive salary and start-up funds are available for this position. Faculty rank and salary will be commensurate with experience. Application Procedure: Interested applicants must submit a complete curriculum vitae, cover letter, a statement of current and future research interests via the online faculty application at <http://www.gwu.jobs/postings/15601>. Review of applications will commence on **July 22, 2013** and continue until the position has been filled. Only complete applications will be considered.

The George Washington University is an Affirmative Action/Equal Opportunity Employer.

Chief Research and Development Officer (CRADO) (Medical & Prosthetics Research) Washington, D.C.

The Department of Veterans Affairs Veterans Health Administration (VHA), Office of Research and Development, is seeking a Chief Research and Development Officer (CRADO). Lead research and development at an award winning organization committed to improving the health of Veterans and the advancement of medicine, in a city where history is made every day! Help fulfill on VHA's mission to discover knowledge, develop VA researchers and health care leaders, and create innovations that advance health care for our Veterans and the Nation.

In this role you will be responsible for policy development, information dissemination, and oversight of the administration of research and the overall direction of the VA research program. The qualified candidate is a licensed physician who has demonstrated expertise and experience in medical care and research of all types, and a high degree of leadership, motivation, self-direction, and initiatives.

Interested applicants may call **1-800-949-0002** for more information.

Apply or refer candidates to:

VAcareers.va.gov/CRADO

VA



U.S. Department of Veterans Affairs
Veterans Health Administration

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U.S. Department of Health and Human Services National Institutes of Health National Cancer Institute Center for Cancer Research

Research Fellow/Staff Scientist position at the NCI-Frederick in the Structural Biophysics Laboratory

The Protein-Nucleic Acid Interaction Section (PNAI), Structural Biophysics Laboratory (SBL) of National Cancer Institute (NCI), National Institutes of Health (NIH), seeks out a NMR spectroscopist to fill a permanent staff position at either a Research Fellow or a Staff Scientist level depending on qualification. The PNAI section is currently at the forefront of developing exciting state-of-art technologies and methods for large RNA structure determination. The SBL operates six high-field NMR spectrometers ranging in frequency from 500 to 850 MHz, five of which are equipped with cryogenic triple resonance probes optimized for studies of macromolecules (proteins and nucleic acids). We also have shared time of the 900 NMR spectrometer located in Bethesda campus. SBL is also equipped with an array of biophysical instruments and operates a small angle X-ray scattering core facility with a fixed access to the beamlines in the Argonne National Laboratory.

We are looking for an enthusiastic, talented and dedicated scientist to join in our exciting cancer research program. The position is located at the NCI-Frederick campus. Candidate must have a Ph.D. and a number of years of relevant post-doctoral experience, theory and practice (pulse programming) of heteronuclear multi-dimensional NMR, structure and dynamics of RNA and proteins, a strong background in physical chemistry and/or physics with excellent analytical and computational skills. Candidate should also have a significant record of first author publications. The candidate shall conduct research of his/her assigned or self-established projects and works cooperatively with the principal investigators and teams of structural biologists and biologists. Candidate will mentor junior staff within the laboratory. This position is not restricted to U.S. Citizens. The salary is determined based on experience and achievements. The performance is evaluated periodically based on the NIH guidelines. Interested applicants should send electronic cv/bibliography, and three letters of references via email to: wangyux@mail.nih.gov; Phone: **301-846-5985**. This position is subject to a background investigation.

The NIH is dedicated to building a diverse community in its training and employment programs. DHHS, NIH, and NCI are Equal Opportunity Employers.

GPCR NMR Faculty Position at the iHuman Institute, ShanghaiTech University (上海科技大学)

The iHuman Institute is established to encourage interdisciplinary research combining chemistry, biology, imaging, and structural biology of the cellular components involved in human cell signaling, with a special focus on G-protein-coupled receptors and their intracellular signaling partners. Academic basic science and applied industry research will be integrated, providing researchers with unique career opportunities. The Institute is recruiting 20 regular tenured and tenure-track faculty to generate an intellectually stimulating environment.

NMR Faculty Position and Qualifications:

The iHuman Institute at ShanghaiTech University is hiring a Professor specializing in the area of solution NMR spectroscopy focused specifically around human G-protein-coupled receptors (GPCRs). The position can be filled either at the level of a tenured senior faculty or a tenure-track junior faculty. The successful applicant will be part of an interdisciplinary research team including mentoring and guidance from Professors Raymond Stevens (GPCR expertise) and Kurt Wüthrich (NMR expertise). The goal is to apply cutting-edge NMR technology towards understanding GPCR structure and function, and to provide new foundations for GPCR-targeted drug discovery. Final decisions on the configuration of the new high-field NMR facility will be made in contact with the successful applicant for the NMR faculty position.

Senior position applicants are expected to be leading scientists in their research area. Junior level position applicants should have a Ph.D. degree in physical or life sciences as well as postdoctoral experience. The successful applicant will be expected to establish an independent, internationally recognized research program and to hire and supervise postgraduate, graduate and undergraduate students.

Computational Biology Faculty Position and Qualifications:

The iHuman Institute at ShanghaiTech University is hiring a Professor specializing in the area of Computational Biology. Researchers interested in the area of computational GPCR biology or GPCR chemistry are encouraged to apply. The iHuman Institute is incredibly strong in the area of x-ray crystallography and NMR of GPCRs, including structure based drug discovery. Computational biologists and chemists interested in understanding cell biology, molecular recognition, and drug discovery should apply.

Senior position applicants are expected to be leading scientists in their research area. Junior level position applicants should have a Ph.D. degree and at least 5 years of experience in the area of computational methods. The successful applicant will be expected to establish an independent, internationally recognized research program and to supervise graduate and undergraduate students.

Initial Research Support Package: The Institute will provide internationally competitive start-up funds plus support of Research Associates and Post-Doctoral Fellows. Research laboratory and office space will be provided to match the research needs. Our faculty will also have access to the research facilities and resources of the Chinese Academy of Science.

Compensation and Benefits: Starting salary will be competitive at the international level and commensurate with qualifications and experience. We offer a comprehensive benefits package including health and retirement benefits as well as on campus housing renting at a reduced rate. The ShanghaiTech University will also sponsor applications of national and local Talent Programs based on individuals' qualification and experience. The iHuman Institute is located in Shanghai, China, which is an exciting and rapidly growing global center of excellence for scientific research.

Application Procedure:

Submit a cover letter, a 2-3 page statement of research interests, a CV and the names and addresses of three individuals who can serve as references to the mail addresses given below:

The iHuman Institute

ShanghaiTech University, Building 8, 319 Yueyang Road, Shanghai 200031, China

Email: iHuman@shanghaitech.edu.cn

Review of applications will start immediately and will continue until positions are filled.

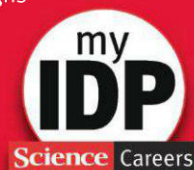
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Investigator position available in the Institute for Translational Medicine, Zhejiang University, Hangzhou, China

Founded in 1897, Zhejiang University (ZJU) is the most comprehensive leading university in China, with distinctive features and high reputations in education and research. In order to promote health science, address growing clinical needs, integrate the multidisciplinary advantages of the university, and bridge the gaps between basic science and clinical practice, ZJU has established the Institute for Translational Medicine (ITM), and is now seeking for the Investigators at all levels in the fields of biomedical and translational sciences, including (but not limited to) Infectious Diseases; Stem Cells, Tissue Engineering and Regenerative Medicine; Neuropsychiatric Disorders; Gastrointestinal Cancer; Heart and Lung Disease; Kidney Disease; Endoscopic Technique; Visual Reconstruction; and Reproductive Health and Safety. Interdisciplinary researchers with interest in medical diagnosis and medical devices are also encouraged to apply.

Applicants should have a PhD or MD degree in the above mentioned areas with postdoctoral training or R&D work experience. Strong track record of publications or unique translational techniques and willingness to work closely with the clinicians in ZJU affiliated hospitals are required. Applicants at the level of associate professor or above with tenure in the overseas Universities are particularly encouraged to apply.

Successful **PIs** will receive quantification- and experience-based compensation, including generous start-up fund (up to 5 million RMB), competitive salary (**up to 800K RMB**) and other health/retirement benefits, and opportunity to purchase an apartment unit (up to 150 m²) at the price much lower than current market value. To apply, please submit your application, including a full CV, cover letter, brief statement of past achievements and future research plan to rzhu@zju.edu.cn before **July 30, 2013**. Both Chinese and non-Chinese nationals are warmly welcomed.



Institute of
Marine and
Environmental
Technology



Assistant Professor: Basic biological processes at the interface between human health and the environment

The Institute of Marine and Environmental Technology (IMET), University of Maryland seeks applications for the position of Assistant Professor (tenure-track). The successful candidate will have a track-record of outstanding research on basic biological processes at the interface between human health and the environment. Potential focus areas include but are not limited to marine natural products discovery, epigenetic effects of toxicants, use of zebrafish as a model system to study development, disease, drug screening or ecotoxicology, fundamental research on biofilms with environmental and human health implications, pathogenic microbes in coastal or terrestrial environments, host-pathogen interactions. Candidates with an interest in practical and biomedical applications of their fundamental research, including interactions with industry, are encouraged to apply. A demonstrated ability to establish a well-funded research program is essential, with very strong preference for candidates with active K99, RO1 or equivalent funded research programs. Primary appointment is at the University of Maryland Baltimore, with a secondary appointment at the University of Maryland Center for Environmental Science. The primary focus of the appointee will be on research; teaching at the graduate level is encouraged. Candidates should explicitly outline the links of their research to human health and environmental applications.

IMET's mission is to study the biology of coastal marine biosystems and ensure their sustainable use, as well as exploit marine-derived systems to improve human health (www.imet.usmd.edu). IMET brings together faculty members from three major University of Maryland research institutions in a dedicated state-of-the-art research facility.

A Ph.D. or equivalent terminal degree is required in molecular biology, microbiology, biochemistry, bioengineering or related fields and candidates should have postdoctoral experience and a strong publication record. Applicants should send the following in a single PDF file to **Russell T. Hill**, Director, IMET; email to hill@umces.edu: (1) detailed curriculum vitae, (2) statement of research interests and goals and (3) names and contact details of three to five references. The position remains open until filled; to receive full consideration, applications should be submitted by **August 1, 2013**.

The USM is an Equal Opportunity, Affirmative Action Employer.

Want to win a rather special prize in Stockholm, Sweden this December?



Winner's paper published in the journal *Science*
\$25,000 dollars grand prize
Awards held in Stockholm in December



Henrik Trygg / Image bank, Sweden

This December a rather special prize will be awarded in Stockholm, Sweden. The journal *Science* and SciLifeLab have come together to recognize and celebrate excellence in PhD research. The *Science* and SciLifeLab Prize has been established to support young scientists at the start of their career.

“Science has never been more exciting and, as leaders in science, we need to support and encourage young researchers today and tomorrow. This prize is a way of doing just that.”

Professor Mathias Uhlén, Director SciLifeLab

The grand prize winner of this major global award will have their paper published in the journal *Science* and receive \$25,000. Three runners up will receive a combined total of an additional \$10,000 in prize money.

The prizes will be presented in Stockholm, Sweden in the middle of December 2013.

To enter

You must be a recent Ph.D. graduate (awarded between January 1, 2011 and December 31, 2012).

Submissions must be in the form of a 1000 word essay, in English, on your thesis, highlighting the significance of its contribution and overall implications in the field. The four submission areas for this prize are: (1) Genomics / Proteomics / Systems Biology (2) Developmental Biology (3) Molecular and Cellular Biology (4) Environmental Life Science.

The deadline for submissions is August 15, 2013. The overall winning essay will be published in *Science*.

For further details and to enter, please go to: www.bit.ly/SciLifeLabPrize

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From technology specialists to patent attorneys to policy advisers, learn more about the types of careers that scientists can pursue and the skills needed in order to succeed in nonresearch careers.



POSITIONS OPEN



INVESTIGATOR AND POSTDOCTORAL POSITIONS in Developmental Genetics

Lecture and Postdoctoral positions are available immediately in the Department of Genetics at The School of Life Sciences Fudan University in Shanghai, China.

We are seeking highly motivated applicants with recent Ph.D. degrees and experience using animal models (zebrafish and mouse), stem cells, and tissue regeneration. An ideal candidate will be creative, industrious, have excellent communication skills and fluency in spoken and written English. Outstanding candidates with postdoctoral experience and excellent publications can be considered as **ASSOCIATE PROFESSOR** positions.

Fudan University offers modern research space and competitive salary/benefit packages. Shanghai is an international metropolitan city offering a multitude of economic, cultural, and athletic activities. To apply, please send a cover letter, a statement of research interests, curriculum vitae, and names and e-mail addresses of three references to **Dr. Tao P. Zhong** (e-mail: taozhong@fudan.edu.cn). Closing date: 31 July 2013.

ENDOWED CHAIR POSITION in The Department of Biology

The Department of Biology at Wheaton College (IL) seeks qualified applicants for the endowed Ruth Kraft Strohschein Chair in Biology. Applicants must have teaching experience, a strong research background in molecular, genetic, or cellular biology with medical application, and a demonstrated understanding of the integration of science and Christian faith. Rank open. Send cover letter that addresses one's mission fit with Wheaton College and curriculum vitae to **Dr. Jennifer Busch**, Biology Department Chair, at e-mail: jennifer.busch@wheaton.edu. Applications will be sent to promising candidates. Deadline for final applications is November 15, 2013, or until the position is filled.

Wheaton College is an evangelical Protestant Christian liberal arts college whose faculty and staff affirm a Statement of Faith and adhere to lifestyle expectations of the Wheaton College Community Covenant. The College complies with federal and state guidelines for nondiscrimination in employment. Women and minority candidates are encouraged to apply. Read more about Wheaton College and its programs at website: <http://www.wheaton.edu>.

MEG POSTDOCTORAL position available at the A.A. Martinos Center, MGH/MIT/Harvard for Studying the Neurophysiology of Autism using MEG. The position will involve investigating local and long-range functional connectivity in autism. The position requires expertise in MEG data analysis methods, and experience working with children. E-mail: tal@nmr.mgh.harvard.edu or further inquiries. Salary is competitive.

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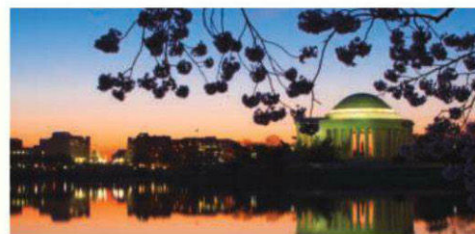
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